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Urinary Excretion of Pyrrole Compounds in Rats Exposed to 2,5-Hexanedione and Co-exposed to 2,5-Hexanedione and N- Acetylcysteine

Sara Bonucci Alves Borges da Costa

Dissertação

Mestrado em Biologia Humana e do Ambiente

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Dissertação orientada pela Professora Doutora Maria Luísa Mateus (DTB/FFUL) e Professora Doutora Deodália Dias (DBA/FCUL)

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"Take up one idea. Make that one idea your life – think of it, dream of it, live on that idea. Let the brain, muscles, nerves, every part of your body, be full of that idea, and just leave every other idea alone. This is the way to success."

(Swami Vivekananda)

The work presented in this Dissertation is inserted under the research project on exposure to n-hexane, developed by Professora Doutora Maria Luísa Mateus.

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I would like to dedicate this dissertation to my grandmother Isabel and my father.

Abstract

n-Hexane is a solvent that has many uses, either in pure form or as a component of the commercial mixture hexane. Highly purified n-hexane is primarily used as a reagent, frequently used in the chemical and food industries, in the formulation of glues and paints and as a degreasing agent and extract solvent.

It is well known that this solvent presents neurotoxic effects, thus, it is very important to study biomarkers, of exposure and/ or effect, as tools of human biomonitoring, acting as indicators of exposure, as well as predictive biomarkers to prevent the occurrence of neurotoxic effects. In this context, it is imperative to understand the mechanism of n-hexane toxicity and identify endpoints that may be selected as predictive biomarkers of neurotoxicity.

The principal aim of this work was to develop accurate procedures to quantify biomarkers in urine of rats exposed to 2,5-hexanedione (2,5-HD), the main metabolite responsible for n-hexane neurotoxicity. This γ-diketone reacts with primary amines of lysine in protein neurofilaments, yielding the formation of pyrrole compounds. However, the formed pyrroles may oxidize and react to other protein nucleophiles, inducing the cross-linking between proteins and causing damage to cellular proteins. The most sensitive proteins to this damage are neurofilaments and other cytoskeletal proteins. In fact, the altered cross-linked proteins aggregate in the distal axon, often just proximal to a node of Ranvier, disrupting the normal physiological cellular activities and causing the neurotoxic effect.

To accomplish the goal of this dissertation, 2,5-HD was administrated in rats during 12 dosages and the pyrrole concentrations were measured, to assess if there were any difference between the control group and exposed rats.

Simultaneously, was studied the role of N-acetylcysteine (NAC) as a possible protective agent of neurotoxic effects evaluating a group of co-exposed rats (2,5-HD+NAC) and a NAC exposed group.

Key-words: n-hexane, 2,5-hexanedione, pyrrole compounds, neurotoxicity, biomarkers

Sumário

A Toxicologia é uma ciência que estuda, entre outros factores, os mecanismos de ação e possíveis efeitos que podem advir da exposição humana a agentes químicos (tóxicos), provocando alterações biológicas no organismo. Esta exposição pode ocorrer através do ar, água, comida, objectos, interferindo diretamente com o ambiente e com o Homem. Neste sentido, o ramo da Saúde Pública aumentou a sua intervenção nesta área, tornando a avaliação da exposição a esses agentes num aspeto de alta importância e prioridade na sua ação, com o intuito de prevenir e/ou minimizar os possíveis riscos/ efeitos na saúde humana, através da criação de protocolos de monitorização biológica ambiental e do estabelecimento de limites (mínimos e máximos) de exposição. O objetivo destes dois tipos de protocolo é aumentar a área de atuação, conjugando a identificação e quantificação dos agentes presentes no local de exposição com a quantificação em diferentes amostras biológicas, para que a avaliação do risco de exposição seja o mais correta possível.

Um dos grupos alvo desse estudo são os solventes orgânicos, principalmente devido às características volatilidade e lipofilicidade que intervêm no mecanismo de absorção e deposição destes solventes no organismo humano. Após a exposição, ocorre a absorção destes químicos que são, imediatamente, transportados pelo sangue, até aos órgãos onde ocorre a sua metabolização (principalmente o fígado), dando origem a metabolitos que, posteriormente, serão degradados e excretados do organismo, provocando o aparecimento de alguns sintomas físicos, tais como dormência, perda de sensibilidade. A gravidade destes sintomas/ efeitos está principalmente associada à via de absorção, sendo as alterações neurológicas as mais frequentes (como por exemplo neuropatias, axonopatias, mielinopatias).

O n-Hexano é um solvente orgânico, altamente volátil e lipofílico, que tem várias aplicações, seja sob a forma pura ou enquanto componente de uma mistura comercial de hexano. A sua forma altamente purificada é primeiramente usada como reagente, sendo as misturas utilizadas nas indústrias químicas e alimentares, na formação de colas e tintas, como desengordurante e solvente de extração.

Da literatura e de estudos anteriores, sabe-se que este solvente é responsável pelo aparecimento de efeitos neurotóxicos (maioritariamente alterações neurológicas), principalmente devido à sua capacidade de acumulação no organismo, sendo, por isso, de grande importância a ação da Saúde Pública na criação/ parametrização de

protocolos de controlo e monitorização. Dentro destes protocolos, surgem os estudos realizados com biomarcadores, de exposição e/ ou de efeito, estando os biomarcadores de exposição associados à quantificação do agente químico e respetivos metabolitos e os biomarcadores de efeito associados à avaliação do potencial dos efeitos resultantes da exposição. Ambos poderão ser utilizados como ferramentas de monitorização humana, que atuem tanto como indicadores de exposição ou como biomarcadores preditivos da ocorrência desses efeitos neurotóxicos. O grau de severidade dos efeitos causados está relacionado com a via de exposição, tempo e grau de exposição, podendo afetar várias partes do corpo, como pele, mucosas das membranas, sistema respiratório, fígado, sangue, sistema reprodutivo e sistema nervoso. Assim, com base no referido anteriormente, percebe-se que é fundamental o conhecimento do mecanismo de toxicidade dos agentes e identificação de *endpoints* que possam ser escolhidos para utilização enquanto biomarcadores de previsão da neurotoxicidade desses agentes.

No caso concreto desta dissertação, o agente em causa é o n-hexano que, após ser metabolizado no fígado dos organismos, origina vários metabolitos, sendo a 2,5-Hexanodiona (2,5-HD) um deles e o principal responsável pelos efeitos adversos que decorrem da exposição ao n-hexano. Posteriormente, esta γ-dicetona é distribuída por vários órgãos/ zonas no organismo, reagindo com os vários componentes lá existentes, dos quais se destacam as proteínas associadas ao funcionamento do sistema nervoso. Esta interação é feita através das aminas primárias do aminoácido lisina nas proteínas (dos neurofilamentos dos neurónios), conduzindo à formação dos aductos pirrólicos, que irão desnaturar a proteína o que, consequentemente, a fará perder a sua função, provocando alterações neurológicas e electrofisiológicas. Por outro lado, os pirróis formados podem também oxidar e reagir com outras proteínas nucleofílicas, induzindo a ligação cruzada entre agregados de proteínas no axónio distal, normalmente próximo de um nódulo de Ranvier, o que também irá interferir com o normal funcionamento das atividades celulares fisiológicas.

Todas as alterações anteriormente referidas estão associadas à acumulação da γ-dicetona em várias partes do organismo que, por métodos analíticos, pode ser quantificada através de uma reação química entre o 4-Dimetilaminobenzaldeído (componente do reagente de Ehrlich) e o anel pirrólico que se forma após contacto da dicetona com as proteínas.

Face a todas estas alterações pode-se ainda falar em possíveis agentes que possam atuar na diminuição e/ ou reversão dos efeitos causados pela dicetona. De entre

essas substâncias está a N-Acetilcisteína que, devido às suas propriedades antioxidantes, tem a capacidade de manter os níveis intracelulares de Glutationo (GSH), que ajuda a reduzir a concentração das Espécies Reativas de Oxigénio (ROS) responsáveis tanto por destabilizações celulares e como pela inibição/ atraso na morte celular. No caso concreto dos compostos pirrólicos, parece atuar reduzindo/ impedindo a oxidação do anel pirrólico, que é o passo determinante na formação dos pirróis, na medida em que provoca rutura e alteração das biomoléculas e células do organismo humano.

Assim, com o intuito de perceber a extensão das alterações causadas pela exposição do organismo à 2,5-HD, através da quantificação dos pirróis, foram estipulados quatro objetivos para esta dissertação: i) desenvolvimento de procedimentos analíticos que permitissem determinar qual o reagente de Ehrlich e respetivas condições de reação (nomeadamente a temperatura de reação) que apresentassem maior sensibilidade na determinação e quantificação dos pirróis; ii) validação do método previamente escolhido como o mais sensível/ adequado, de acordo com normas e parâmetros já definidos na literatura; iii) determinação da influência da administração (por injeção) de 2,5-HD na concentração dos pirróis, em amostra de urina de ratos *Wistar* e iv) teste do efeito de proteção da NAC face à formação dos aductos pirrólicos, enquanto agente antioxidante que atua na redução e/ou eliminação dos efeitos resultantes da exposição ao metabolito 2,5-HD.

Para o primeiro objetivo, utilizou-se um método espectrofotométrico para quantificação dos pirróis, com base numa reação colorimétrica entre soluções-padrão, de concentrações conhecidas, e o reagente de EH. Experimentalmente foram comparados dois reagentes diferentes, um preparado com trifluoreto de boro e outro com ácido clorídrico, à temperatura ambiente e a 45ºC. Estes estudos foram realizados com o intuito de perceber qual o reagente de EH e a temperatura que permitia uma maior sensibilidade do método e, consequentemente, uma melhor aproximação da verdadeira concentração dos pirróis nas amostras de urina analisadas. Dos resultados obtidos, concluiu-se que o melhor método a utilizar é o reagente de EH com ácido clorídrico, à temperatura ambiente, devido à maior sensibilidade, simplicidade e menor toxicidade, estando esta última característica associada à ausência de trifluoreto de boro, uma substância bastante tóxica.

Após a escolha do método a utilizar, procedeu-se à validação, seguindo parâmetros definidos para métodos internos de ensaio em análise química, tais como: linearidade, gama de trabalho, limites de deteção e quantificação, sensibilidade, precisão

e exatidão. Nesta parte foi avaliada a curva de calibração determinada para o reagente de EH com ácido clorídrico à temperatura ambiente. Tanto a linearidade como a gama de trabalho são parâmetros que foram analisados estatisticamente, comparando um valor calculado utilizando os resultados obtidos com um valor já definido na literatura e, em ambos os casos, os resultados estavam bem ajustados. De acordo com os resultados obtidos, 2,4966 nmol/ mL é a menor concentração que pode ser detetada nas amostras (limite de deteção) e 3,3810 nmol/ mL a menor concentração possível de ser quantificada utilizando a curva previamente determinada. 0,01876 é um valor que está associado à capacidade do método em distinguir pequenas diferenças entre as concentrações dos analitos. Por fim, dentro da precisão temos a repetibilidade e a precisão intermédia que permitem avaliar a reprodutibilidade do método em condições de variabilidade, tais como laboratórios, analistas, equipamento, tipos de reagentes e duração. A exatidão foi o único parâmetro que não foi avaliado neste trabalho experimental por não haver nenhum valor teórico que se pudesse utilizar para comparação.

O cumprimento do terceiro objetivo foi feito através da comparação da concentração dos compostos pirrólicos entre grupos de ratos expostos a diferentes doses de 2,5-HD. As doses foram injetadas por via intraperitoneal, em dias alternados, durante um total de 12 administrações (doses), das quais foram avaliadas as doses 1,4,8 e 12, que estão associadas aos dias de recolha de urina. Da comparação dos resultados obtidos, para o grupo controlo (injeção intraperitoneal de soro fisiológico) concluiu-se que a primeira dose resultou na distribuição da 2,5-HD pelos tecidos, pois os níveis deste composto na urina dos ratos expostos são bastante superiores aos níveis apresentados pelos ratos do grupo controlo. Quanto às outras doses, não foi possível observar se houve diferença significativa, pois i) existe uma grande variabilidade entre os animais, responsável por grandes desvios na análise estatística e ii) devido ao possível surgimento do estado estacionário, em que a determinada altura, a taxa de absorção se torna igual à taxa de eliminação, tornando a concentração dos pirróis constante.

Para o quarto e último objetivo desta dissertação, os ratos foram co-expostos à 2,5-HD (injeção intraperitoneal) e NAC (adicionada à água de beber), com o intuito de comparar as concentrações nos dois grupos para testar o possível efeito protetor da NAC, face à injeção de 2,5-HD. Para a primeira dose administrada, foi possível observar que existe um fator protetor quando se adiciona NAC na água de beber que é

dada aos ratos, pois a concentração de compostos pirrólicos do grupo co-exposto é inferior à concentração destes mesmos compostos na exposição única a 2,5-HD. Com a continuação da exposição para as restantes doses, o efeito da NAC foi-se tornando menos evidente, o que poderá estar associado ao facto da dose de NAC administrada nos ratos não ser suficiente para reduzir o efeito da exposição repetida à 2,5-HD.

Palavras-chave: n-hexano, 2,5-hexanodiona, compostos pirrólicos, neurotoxicidade, biomarcadores

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List of Abbreviations

2,5-DMP – 2,5-Dimethylpyrrol

2,5-HD - 2,5-Hexanedione

BEI – Biological Exposure Index

CNS – Central Nervous System

CV – Coefficient of Variation

CYP450 – Cytochrome P450

DMAB – 4-Dimethylaminobenzaldehyde

EH – Ehrlich's Reagent

EH-A – Ehrlich's reagent with boron trifluoride

EH-B – Ehrlich's reagent with hydrochloric acid

F – Fisher-Snedecor test

GSH – Glutathione

LD – Limit of Detection

LQ – Limit of Quantification

NAC – N-acetylcysteine

NF – Neurofilaments

NF-H – Neurofilaments-High

NF-L – Neurofilaments-Low

NF-M – Neurofilaments-Medium

OLAARP – Oxidized Lipid/ Amino Acid Reaction Products

PG – Test-value

PNS – Peripheral Nervous System

ROS – Reactive Oxygen Species

RT – Room Temperature

SD – Standard Deviation

TLV – Threshold Limit Value

CHAPTER 1

INTRODUCTION

CHAPTER 1 – INTRODUCTION

1 GENERAL INTRODUCTION

Toxicology, as a science, is the study of adverse effects, caused by xenobiotic, having evolved since old medicines/ poisons (Old Toxicology) for the study of Molecular Biology (Modern Toxicology), studying the mechanisms of action and the possible effects that could come from human exposure to toxins, resulting in an acute effect (exposure to high concentrations, yet occasional) or chronic effect (repeated exposures, yet at low concentrations) (Casarett and Klaassen, 2008). Simultaneously through evolution and development of Toxicology, appears the evolution of Man that, while a living being, and due to the development of even more complex activities, with the goal to guarantee his survival, is subjected to various risks arising from the environmental factors involved, such as psychological, accidental, physical and/ or chemical. The exposure to any of these factors can occur in many ways, interfering directly with environment and with Man, principally the chemical agents, at which a population is exposed on a day-to-day basis, through the air, the water and the food (Amorim, 2003).

Thereby, the assessment of exposure to these chemical agents has turned into an important aspect of Public Health, in an attempt to prevent and/ or minimize the possible risks/ effects on human health, arising from the interaction between chemical agents and human organism, before an illness (intoxication) installs (Amorim, 2003). This exposure assessment was to draw up plans/ protocols for human monitoring, from exposure to chemicals, based on a routine evaluation and interpretation of biological and/ or environmental parameters (Amorim, 2003). The identification and quantification of those effects are parameters that integrate the new concept of Toxicology, with effect since 1975, associated to security assessment and possible risks resulting from the exposure to chemical agents (Casarett and Klaassen, 2008).

2 CONTROL AND EXPOSURE ASSESSMENT

The toxic effects of a chemical agent are related with its ability to generate a certain biological effect, that only occurs when the agent, or its metabolites, are on the minimal necessary conditions (concentration and period of time) to cause toxic manifestations (Casarett and Klaassen, 2008). The risk assessment resulting from exposure in the workplace is the main objective of Occupational Toxicology, to protect the workers exposed to potentially hazardous chemicals (Winder and Stacey, 2005).

The development of this branch of Toxicology has been increasing due to the rise in the production of organic chemicals, with an annual production of hundreds of millions of tons, responsible for a huge part of the effects that appear in the population exposed to them (Winder and Stacey, 2005). This rise led to the need to establish permissible exposure levels (exposure limits), characterized by the concentration at which almost all the workers may be exposed, day after day, without adverse effects on their health (Amorim, 2003; Casarett and Klaassen, 2008). These exposure limits may be open to possible alterations, being the control of those variations made by two types of monitoring: the Environmental Monitoring and the Biological Monitoring. The Environmental Monitoring consists of the identification and quantification of the agents (chemical) present in the air at the location of exposure, being their exposure limits called Threshold Limit Values (TLV). Biological Monitoring is associated to the quantification of the agent concentration, or its metabolite, in several biological samples like blood and urine, through the comparison between obtained value and the referenced values in literature, being these limits designated by the Biological Exposure Index (BEI) (Casarett and Klaassen, 2008).

Both monitoring have a mutual objective that consists in the risk assessment of exposure in health, fulfilled through different evaluation parameters. However, in the achievement of this purpose the Biological Monitoring presents an advantage over the Environmental, based on an evaluation of the chemical in the certain target organ, allowing a more effective determination of protection measures in the exposure to chemicals (Casarett and Klaassen, 2008).

The biological indicators or biomarkers that are used to characterize biological/ biochemical/ molecular markers (Costa, 1996), are defined as all the substances, or

subproducts of their metabolism, as well as any early biochemical change, which can be measured in biological fluids, tissue or exhaled air allowing the evaluation on the exposure intensity and health risk. Among the objectives of their use are i) exposure evaluation, absorbed quantity or internal dose, ii) evaluation of chemicals effects and iii) evaluation of individual susceptibility. This classification divides the biomarkers (Figure 1.1) into three classes, exposure, effect and susceptibility, respectively (Amorim, 2003).

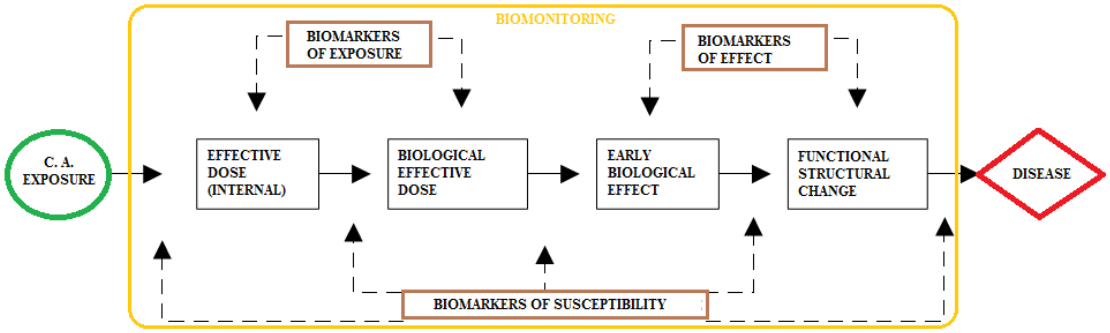

Figure 1.1: Control and exposure assessment through biomonitoring. Schematic representation of the steps from biomonitoring, since exposure to chemical agents (C.A.) until the onset of the disease. (Adapted from Amorim, 2003).

From the classes of biomarkers referred, those whose function, in respect to the risk assessment, is best defined are biomarkers of exposure and effect (Lowry, 1995).

The biomarkers of exposure allow the quantitative measurement of both a chemical or its metabolites, such as reversible biochemical change, in biological fluids that present signs of exposure to the agent (Amorim, 2003; Lowry, 1995). Must be specific, detectable in vestigial quantities, available for non-invasive techniques and quantitatively related with previous exposures (Costa, 1996). They also present a great importance in the determination of the nature of exposure to the chemical, its bioavailability and, in some cases, the potential presented by the chemical for production of adverse effects (Lowry, 1995).

The biomarkers of effect reflect an evaluation of the potential of adverse effects caused by a chemical, through the evidence of the interaction between the chemical and the biological receptors. These biomarkers must still reflect early biochemical modifications that precede structural or functional changes (Amorim, 2003; Costa, 1996; Lowry, 1995).

The role of biomarkers of susceptibility, despite not very well defined, also contributes to the risk assessment, identifying, in one population, the individuals that have a difference, genetic or acquired, in the susceptibility due to the exposure to the chemicals. This allows the knowledge about which external factors can raise, or decrease, the individual risk in the development of the organism response, during chemical exposure. This is because, besides similar exposure, genetic differences in metabolism may produce different doses in the target organ, interfering with the responsiveness of the organism to the agent upon exposure (Amorim, 2003; Costa, 1996).

For the quantitative determination of biomarkers be possible, it is necessary to know two main characteristics used in the characterization of the chemical agent: toxicokinetics and toxicodynamics. The toxicokinetics is the study of the metabolizing process of the chemical (absorption, distribution, accumulation, metabolism and excretion), being its study associated to the determination of the chemical substance, or any of its metabolites, in a biological medium. The toxicodynamics is related with the mechanism of action (in the target organ) of the chemical, being its study applied in cases of measurement of biological changes in the organism caused by the chemical exposure, to identify what are the effects resulting from that change (Amorim, 2003).

2.1 ORGANIC SOLVENTS

Organic solvents are chemical substances that have the ability to dissolve, dilute or disperse one or more substances that are insoluble in water. They constitute a big and diverse chemical group, still in development (Casarett and Klaassen, 2008), quite relevant in industry (Williams et al., 2000), for example as constituents of paints, varnishes, lacquer, aerosol products (sprays), adhesives, intermediates in chemical syntheses, fuel and fuel additives (Casarett and Klaassen, 2008).

Besides specific properties of each compound, based on a set of characteristics, such as number of carbon atoms, number and kind of chemical bonds, chemical configuration and the presence of functional groups (Winder and Stacey, 2005), this group of compounds presents two common characteristics: volatility and lipophilicity (Casarett and Klaassen, 2008).

Both properties are responsible for the absorption and deposition of the solvent in the organism, varying according to molecular weight. In general, the lipophilicity of solvents increases with increasing numbers of carbon and/or halogen atoms, while volatility decreases. These properties also vary according with the charge of the molecule, particularly its absence, that, coupled with low molecular weight, make inhalation the major route of solvent exposure and providing a ready absorption across lung (inhalation route), gastrointestinal tract (ingestion) and skin (dermal exposure) (Casarett and Klaassen, 2008).

Once absorbed, solvents may be transported by the blood to the organs where biotransformation may occur, resulting in the formation of metabolites that will be degraded and excreted from the organism. In the case of absorption following ingestion or dermal exposure, organic solvents are absorbed into the venous circulation, making the transport to the liver faster, resulting in faster metabolism, degradation and excretion. Absorption following inhalation route happens via the alveoli in the lungs. Due to the lipophilic characteristics of the solvents, the solvents cross to the blood, spread in the organism, making the transport to the liver slower and consequently decreasing the speed of metabolism, degradation and excretion of absorbed chemicals (Williams et al., 2000). Thus, it can be stated that there is a relationship between the absorption route and the severity of the adverse effects, resulting from the exposure to solvents, depending on the effects from factors like solvent toxicity, exposure route, frequency and volume of inhaled air, individual susceptibility, interaction with other chemicals (Casarett and Klaassen, 2008; Williams et al., 2000).

Among the factors defining the severity of effects, the frequency of exposure is one of the most important because of the distinction between acute or chronic effects. Occasional acute exposures lead to effects very different from those caused by more extended exposures, yet at similar concentrations (Casarett and Klaassen, 2008; Williams et al., 2000). Of the main effects, the one of particular importance is neurotoxicity which is associated to alterations in the central and peripheral nervous system (Williams et al., 2000).

3 NEUROTOXICITY

Neurotoxicology is an area that connects neurosciences and toxicology and plays a very important role in the understanding of the functions of the nervous system. The field of neurotoxicity is important in our understanding of how the nervous system work, how environmental factors may play a role in system disorders and how to intervene to prevent damage and restore affected/ loss functions (Costa, 1996; Williams et al., 2000).

By definition, neurotoxicity is defined as any permanent or reversible effect on the structure or function of the central and/ or peripheral nervous systems by a biological, chemical or physical agent (Costa, 1996; Spencer, 1990; Winder and Stacey, 2005). However, its evaluation could have some limits due to the complexity of nervous system function, the multiple nature of neurotoxic events, the variability and inaccessibility of cellular and molecular locals that compose this system as well as the later expression of neurotoxic effects, after prolonged exposures or even after a latency period (Amorim, 2003).

Nervous system, one of the main human systems, is responsible for the control, coordination and regulation of corporal activities, through reception, transmission and integration of the information that allows the reaction and adaptation to the surrounding environment (Kulig et al., 1996). It could be divided into two subsystems: the Central Nervous System (CNS), comprising mainly the brain and spinal cord, and the Peripheral Nervous System (PNS), comprising all other components, including sensory and motor nerves (LoPachin and DeCaprio, 2004; Winder and Stacey, 2005). This distinction is important because some toxins appear to target only the central or the peripheral nervous system, but not both (Williams et al., 2000).

The main structural unit of the nervous system is the neuron, also called nervous cell, which connects with other neurons and is supported by auxiliary cells. The neuron exists in several shapes and sizes and with different functions, however they present a common morphological structure-based, composed by dendrites, cellular body and axon, shown in Figure 1.2 (LoPachin and DeCaprio, 2004; Williams et al., 2000).

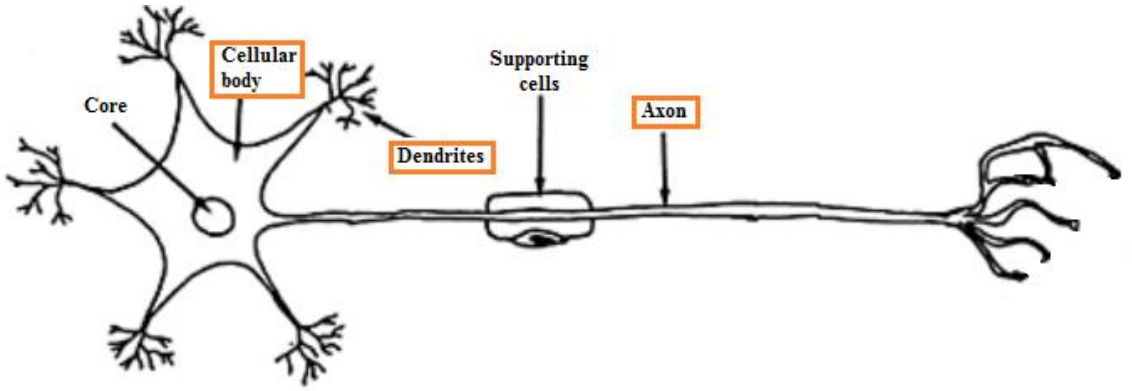

Figure 1.2: Schematic representation of the morphological structure of neuron. The oranges rectangles are highlighting the common structures in neurons. (Adapted from Winder and Stacey, 2005).

Due to the importance of neuron functions to the correct development and organism regulation, any factor that cause changes in its normal functioning, will induce functional and/ or morphological changes, that could reflect, mainly, in the decreasing of the initials capabilities of the system (LoPachin and DeCaprio, 2004). Following this idea, and considering that neurotoxins could affect separate parts of the nervous system, the study about the mechanism of action of neurotoxins, namely the target, becomes quite relevant and important. A more specific understanding allows a more specific treatment with the aim of decreasing and/ or reversing the changes caused by the chemicals (Casarett and Klaassen, 2008).

The response of the nervous system to chemical agents is based on a set of aspects, including i) the maintenance of a biochemical barrier between the brain and the blood, ii) the importance of the high energy requirements of the brain, iii) the maintenance of an environment rich in lipids, iv) the transmission of information across extracellular space at the synapse, v) the distances over which electrical impulses must be transmitted, coordinated and integrated and vi) the development and regenerative patterns of the nervous system (Casarett and Klaassen, 2008).

From the interaction between a certain chemical and a certain target can result many pathologies, also designated by pathologic responses. The four main targets of nervous system are: the neuron, the axon, myelinating cells and the neurotransmission system. The respective pathologies are designated by neuropathies, axonopathies, myelinopathies and toxicity associated to neurotransmission (Figure 1.3) (Casarett and Klaassen, 2008).

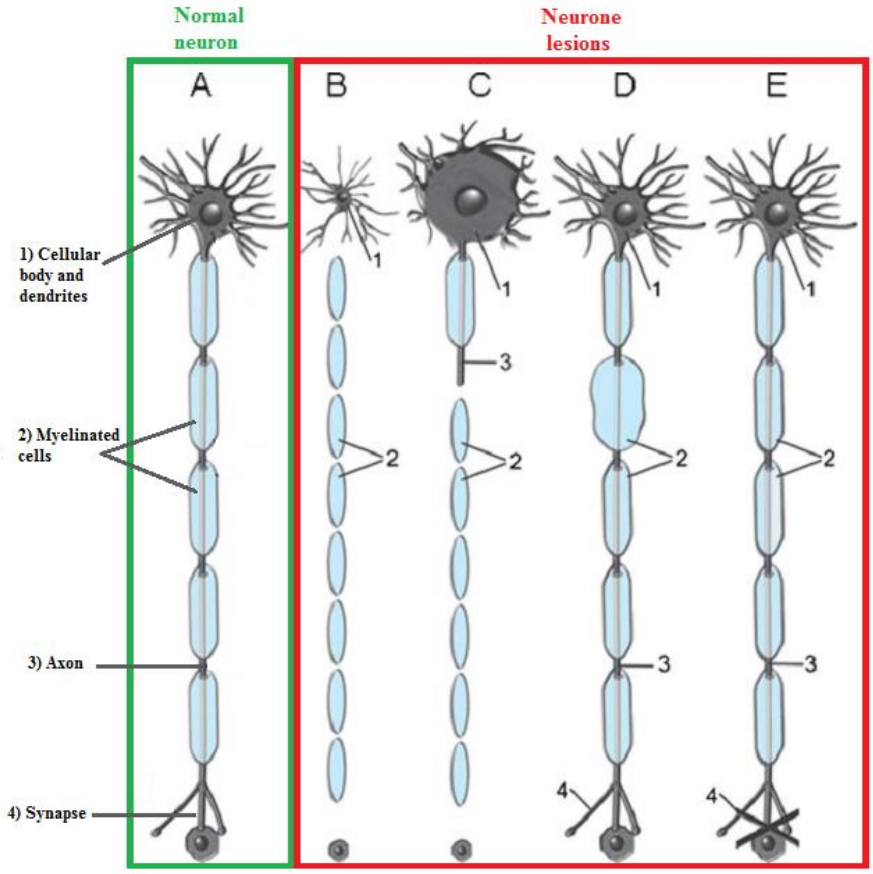

Figure 1.3: Neuronal patterns resulting from the chemicals action. A. Normal neuron; B. Neuropathy; C. Axonopathy; D. Myelinopathy; E. Toxicity in transmission (Adapted from Casarett and Klaassen, 2008).

The neuropathies (Figure 1.3-B) are caused by the specificity of a given toxin to the neurons, or to any of its groups, producing changes that, in this case, are responsible for the loss of cellular body and all its associated processes, with no potential for regeneration. In cases of severe exposure, the neurons may die (Casarett and Klaassen, 2008).

When the target is the axon, the pathologies are called axonopathies (Figure 1.3-C) and the toxins are responsible for axon degeneration and consequent myelin sheath damage, maintaining the cellular body intact. In this setting, there is potential for regeneration and recovery from the toxic injury as the axonal stump sprouts and regenerates (Casarett and Klaassen, 2008).

Myelinopathies (Figure 1.3-D) are morphologic changes that occur on the myelin sheath that coats the axons. Myelin function is based on a proper electrical isolation of neuronal processes and, in its absence, the transmission of impulses along

axons may change, for example reducing the rate of transmission, which can affect the normal functioning of nerve cells, leading to the development of myelinopathies (Casarett and Klaassen, 2008).

The last figure (Figure 1.3-E) illustrates a change that occurs in the transmission of nerve impulses at synapses. In this case, the changes are taking place in the intracellular signaling mechanisms, keeping the structure of the neuron intact. They also may lead to a decrease in the capabilities/ functions of the nervous system, mainly through behavioral changes, since the transmission does not occur properly (Casarett and Klaassen, 2008).

3.1. EVALUATION OF NEUROTOXIC EFFECTS

The nervous system is one of the most complex systems that exist in the human organism, due to its many constituents, that after certain stimuli produce different responses. This complexity makes it difficult to study the toxic effects that appear after chemical exposure, because in different targets, the same chemical could cause different responses, making necessary the choice of an adequate method of study. Kulig (Kulig et al., 1996) argued that the choice of the approach to be followed and the methods to use in a particular investigation depend on the scientific question that underlies the study.

Following the guiding thread of Kulig's idea, Tilson (Tilson, 2000) stated that, for the neurotoxic studies in humans, behavioral studies in animals should be used, because they allow a good evaluation of neurobiological functions (sensory, motor, autonomous and cognitive functions) that are affected in humans, during chemical exposure.

Behavioral studies are techniques generally used in a tiered-testing scheme, based on decision points including in each stage of evaluation that allows the investigator to know whether or not available information is sufficient for concluding if a chemical should be considered a neurotoxin or not (Tilson, 1993). If, on the first tier, a chemical was observed to be a neurotoxin, the second tier is necessarily required, corresponding to the chemical characterization (Tilson, 2000). Characterization studies might be based on results from the first tier, already existing published data, or on new toxicological data suggesting that the chemical may pose a human neurotoxic risk (Tilson, 2000). The high degree of recurrence of this type of research is justified by its

greater sensitivity to other indicators of neurotoxicity and its early observation, allowing an observation of effects during exposure.

Based on this, Tilson (Tilson, 1993) presented one of the possible models to assess toxicity, which comprises two stages: i) toxic identification and ii) characterization of resulting effects from the toxic exposure. First stage, which consists of the initial evaluation of the neurotoxic potential of the chemical (Becking et al., 1993), is based on less expensive, simple and rapid methods, such as sensorimotor functions, locomotor activities and neurological signs. In the second stage, the methods chosen are more complex and specific, because they are used to specifically assess sensory and motor dysfunctions and to quantitatively measure chemical changes, which may be induced mainly in cognitive functions, allowing a better characterization of the nature and the mechanisms of the effects induced by the toxins (Becking et al., 1993; Tilson, 1993).

Later, Kulig (Kulig et al., 1996) justified the importance in using behavioral studies, stating that direct observation of an animal behavior, during chemical exposure, is one of the simplest methods to document clinical signs of neurological and behavioral impairment and a logical starting point for investigating the potential neurotoxic effects of a chemical whose data about neurotoxicity are incomplete or nonexistent.

4 N-HEXANE

n-Hexane is an organic solvent, which is very volatile, colorless and with an unpleasant odor, fat soluble, easy evaporation, highly inflammable and is possible to accumulate in the body (cumulative effect) (Cheng et al., 2012; Song et al., 2012; U.S. Department of Health and Human Services, 1999; Zhang et al., 2013). It can be used in pure form, particularly as a laboratory reagent or as component in a mixture, being its applications, in this last case, almost all associated with the industry, as a solvent or a thinner (Jorgensen and Cohr, 1981). Among several industrial applications are the production of tires and impregnation of materials in the rubber industry, production of tablets in the pharmaceutical industry and applications for the perfume industry. n-Hexane can still be used in product adhesives, cleaners, textiles, furniture manufacturing, printing industry, shoe-making, solvent cements and thinners and, in minor amount, in crude oil and natural gas (Fedtke and Bolt, 1987a; Integrated Risk

Information System, 2005; Jorgensen and Cohr, 1981; U.S. Department of Health and Human Services, 1999).

n-Hexane can be present in the air, in food and water and in objects/ materials that could contact directly with humans, which means the absorption of this solvent could occur in many ways. If it is in the air that we breathe, it can enter the body through the lungs; if it is in food or drinking water, it enters by ingestion through the stomach and intestines; if we come into contact with it, it enters through the skin. How much n-hexane enters the body depends on the exposure time, the amount at which humans are exposed and the exposure route (U.S. Department of Health and Human Services, 1999). From the possible exposure routes, inhalation is the most studied, because it is the most likely form of contamination. It occurs in the lungs, through the process of passive diffusion in membranes of epithelial cells. After, it enters into circulation, being transported to the liver, where it is converted, by metabolic processes, into its metabolites, which are distributed to various body organs, mainly the brain, liver and kidney (Integrated Risk Information System, 2005).

Depending on the route of exposure, as well as the time and amount of exposure, the effects that n-hexane cause are various, affecting many parts of the organism, such as skin, mucous membrane, respiratory system, liver, blood, reproductive system and nervous system (central and peripheral) (Jorgensen and Cohr, 1981). From these neurotoxic effects are, specifically, the progressive sensorimotor neuropathy beginning with prickling, burning and loss of sensation, first in the feet, moving gradually up the legs and eventually involving hands (Genter St Clair et al., 1988).

The first neurotoxic effects associated with n-hexane exposure were noticed in Japan, in the mid-60, in workers from the shoes industry. Workers of this industry were exposed to several chemicals, beyond n-hexane, complicating the identification of symptoms deriving from n-hexane exposure. It was only in 1969 that Yamamura (Integrated Risk Information System, 2005; Yamamura, 1969) began a study that allowed the determination of specific symptoms resulting from the n-hexane exposure.

Following this idea, n-hexane was characterized as a neurotoxic compound, making necessary the development of protection measures that could reduce exposure and, consequently, the neurotoxic risk. To this end, the toxicokinetics and

toxicodynamics of n-hexane were characterized to try to determine the target and mode of action of n-hexane and its metabolites (Casarett and Klaassen, 2008).

4.1 N-HEXANE BIOTRANSFORMATION

n-Hexane shares the common property of organic solvents, high volatility, causing exposure occurrence mostly in the form of vapor, via inhalation (U.S. Department of Health and Human Services, 1999). It is absorbed by the lungs, being rapidly transported to the other organs of the body.

The metabolism of n-hexane occurs, mainly, in the liver, where the toxin undergoes a set of reactions of hydroxylation and hydrogenation, shown in Figure 1.4. It starts with a hydroxylation of n-hexane, by the action of mixed function oxidases to form either 1- or 3-hexanol, in a detoxification pathway, or 2-hexanol in a bioactivation pathway. Through the bioactivation pathway, 2-hexanol is converted to 2-hexanone and 2,5-hexanodiol. Both of these metabolites are then further metabolized to 5-hydroxy-2-hexanone, through a new oxidation reaction, that, by a hydroxylation reaction, generates 4,5-dihydroxy-2-hexanone. By oxidation of this metabolite, through acid hydrolysis of urine, or oxidation of 5-hydroxy-2-hexanone, 2,5-HD is formed. 2,5-HD is the final compound of n-hexane biotransformation, being considered the major toxic metabolite produced in humans, responsible for the production of effects associated to n-hexane exposure. After this, it is important to clarify that the difference between bioactivation and detoxification pathways is associated with the toxicity of the metabolite that is being studied, i.e., if the metabolite is the most toxic that results from the metabolizing, the pathway that will end in its formation will be called bioactivation. If, in reverse, the metabolite is not the most toxic, it will be formed through a detoxification process. That is why here (for the n-hexane) was studied the metabolism through the bioactivation pathway.

At the end of metabolizing, the excretion/ elimination of the resulting products of n-hexane exposure can occur in different ways, being the urine the main form of elimination from the body (Integrated Risk Information System, 2005; U.S. Department of Health and Human Services, 1999) and, consequently, the most studied.

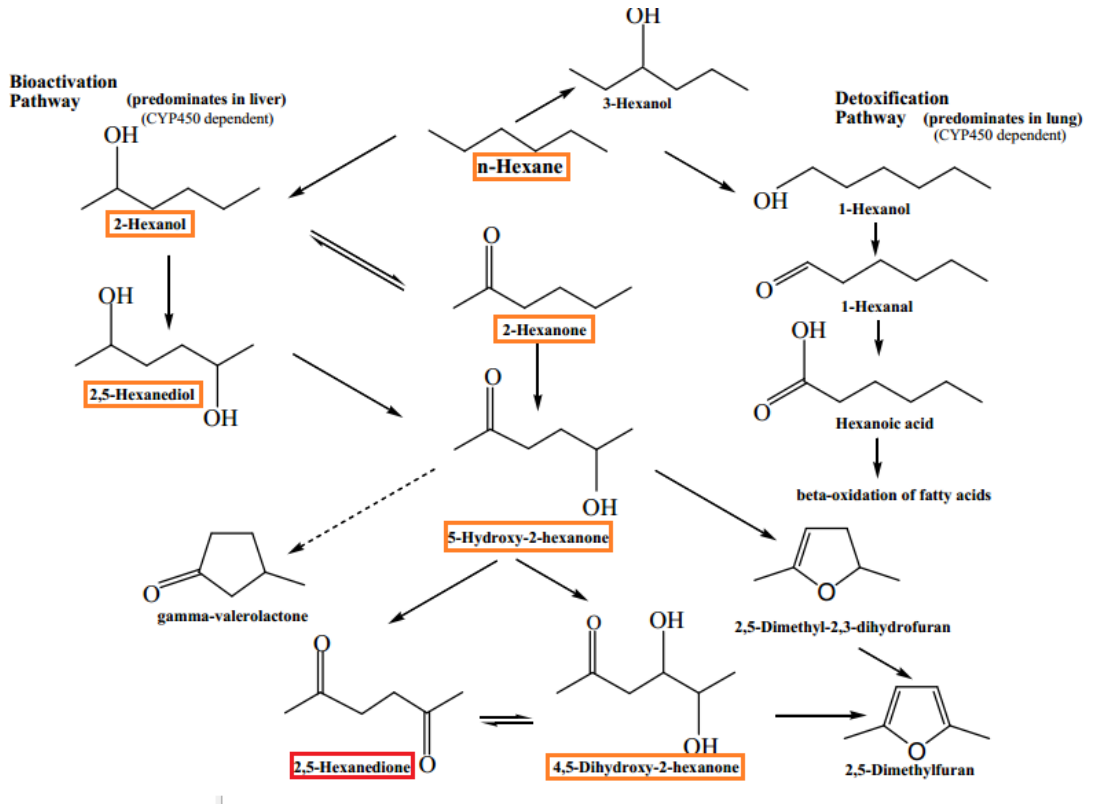

Figure 1.4: Biotransformation of n-hexane. Compounds marked with orange rectangles indicate the route of metabolism of n-hexane-associated neurotoxicity. With red color is marked the major metabolite responsible for n-hexane associated neurotoxicity. (Adapted from Integrated Risk Information System, 2005).

The metabolism of n-hexane to 1-, 2- and 3-hexanol is carried out by, at least, four enzymes, whose identification was not possible by kinetic data collected in some studies. However, from the observation of requirements from the reactions catalyzed by these enzymes, mainly the presence of NADPH, some authors may suggest that the enzymes responsible for the metabolizing of n-hexane could be Cytochrome P450 (CYP450) isozymes (Crosbie et al., 1997; Soriano et al., 1996). Further studies reinforced this idea, admitting that CYP450 catalyzes the initial steps of n-hexane metabolism, the detoxification and bioactivation pathways, being considered CYP450 dependent reactions. So, these enzymes are the ones that, acting directly on n-hexane, are going to metabolize the toxic, through bioactivation pathways, yielding, among others metabolites, 2,5-HD, responsible for the neurotoxicity associated to n-hexane exposure (Integrated Risk Information System, 2005).

2,5-HD is also considered a consequence of exposure to methyl-n-butyl-ketone, another industrial solvent whose exposure results in similar effects to those produced by

n-hexane exposure (Abou-Donia et al., 1982; Perbellini et al., 1993; Pyle et al., 1992; Tshala-Katumbay et al., 2009). The similarity in the resulting neurotoxic effects indicates a common mechanism of toxicity, essentially related with oxidation and production of the toxic metabolite, the 2,5-HD (Pyle et al., 1992). This neurotoxic effect is thought to be attributed to the reaction between this metabolite and functional (NH_2)-groups of axonal proteins under formation of substituted pyrroles adducts (Fedtke and Bolt, 1987b), point that will be discussed later.

4.2 MECHANISM OF ACTION AND PATHOLOGY

Neurons are cells of the human body that, when exposed to 2,5-HD, have a high degree of structural alteration, mostly due to the degradation of their constituent proteins, at axon level (Song et al., 2012). These cells, under physiological conditions, are capable of maintaining a low level of autophagic activity that consists of the elimination of some damaged organelles and proteins. This cellular process is important to maintain the homeostasis of axons (Song et al., 2012). In the presence of 2,5-HD, autophagy levels are substantially decreased, leading to the gradual accumulation of proteins, organelles and aberrant structures in the membrane of the axon terminals. This accumulation is confirmed by observation of axonal swellings (Song et al., 2012) which, together with axonal atrophy, retraction of the myelin sheath of the nodes of Ranvier and segmental demyelination (Wang et al., 2008), characterize the most frequent pathologies designated axonopathies.

The emergence of these effects in workers exposed to n-hexane led to the development of several studies, to try to discover the mode of action of n-hexane, specifically the mechanism by which 2,5-HD causes axonopathies.

Initially, many authors reported that the structural changes observed in axons, atrophy or degeneration, were associated with the reactivity of γ-diketones, stating that the 2,5-HD was, clearly, the diketone responsible for axonopathies (Lopachin and Decaprio, 2005; LoPachin and DeCaprio, 2004; Tshala-Katumbay et al., 2009). Other studies revealed that axonopathies may be caused by several factors, such as changes in cytoskeletal proteins of axons that occur in the neurofilaments (NF), which can achieve a reduction in the diameter of the axons and consequent decrease in conduction velocity

of the nerve, causing neurological and electrophysiological modifications (Lopachin and Decaprio, 2005).

For the case of NF, since they are the primary cytoskeletal components in the large and myelinated fibers which are highly vulnerable to n-hexane and related neurotoxins (Decaprio and Fowke, 1992), there are many studies that prove that the change in its structure or its normal functioning can be reflected in the pathologies described above. The NF, described as the intermediate filaments in mature neurons (Wang et al., 2011), are the most abundant components of the neurons (Wang et al., 2011) that have an essential role in the establishment and maintenance of the maturation of the axons caliber (Chiu et al., 2000). Structurally, the filament itself is composed by three subunits proteins (NF-Low (NF-L), NF-Medium (NF-M) and NF-High (NF-H)), that copolymerize via coiled-coil interactions of the rod domains. According to an immunohistochemical analysis, NF-L represents the core and the tail domains of NF-M and NF-H form the peripherally oriented filament arms (Muma and Hoffman, 1994). These latter structures mediate NF-NF interactions (Decaprio and Fowke, 1992). Each of the subunits has its own molecular weight: NF-L has 68kDa, NF-M 95kDa and NF-H 115kDa (Chiu et al., 2000; Muma and Hoffman, 1994; Wang et al., 2011).

The contribution of NF to the development of axonopathies can occur in two ways: through the change of axon caliber or through the change of NF protein constituents, both after exposure to the diketone 2,5-HD (Chiu et al., 2000; LoPachin and DeCaprio, 2004).

The decrease in axon caliber of NF is a mechanism that is not well understood, but there are studies that claim that this mechanism is based on a decrease in the interfilamentar space through dephosphorylation processes, associated with some amino acids residues (lysine, serine and proline), which, when phosphorylated, are responsible for the maintenance of that interfilamentar space (Chiu et al., 2000) and respective thickness of axon caliber. Subsequent studies have established a model, stating the axon caliber is regulated by domains of NF-M and NF-H, which form long bridges, enabling determination of interfilamentar space, defining the various spaces and the specific size of the axons.

Changes at the level of NF protein constituents pass through adduct formation, which are irreversible covalent bonds formed by linking a chemical and a biological

molecule (Lopachin and Decaprio, 2005; Tornqvist et al., 2002; Wang et al., 2011). The toxicity of the chemical agent is associated only with the time that the formation of adducts "breaks" the structure in question or destroys the function of the macromolecule (Cheng et al., 2012). In the specific case of n-hexane, the reaction of its metabolite, γ-diketone 2,5-HD, with neurofilamental proteins (only about 5%) (LoPachin and DeCaprio, 2004), leads to the formation of pyrrole adducts of 2,5-dimethylpyrrol (2,5-DMP) (LoPachin et al., 2005), through its interaction with the ε-amino groups of lysine residues. This leads to the formation of misfolded proteins that, besides contributing to the decrease of axon caliber, may affect normal growth and development of axons, by reducing the handling capacity of the proteins that interact with the polymeric cytoskeleton (LoPachin and DeCaprio, 2004; LoPachin et al., 2005; Wang et al., 2011). The determination of proteins and/ or pyrroles formed in biological samples, such as serum, in study cases of n-hexane exposure, may allow inference of a possible presence and degree of injury in neurons (Song et al., 2008).

A third hypothesis used to try to justify the development of axonopathies focuses on the role of cytoskeletal proteins. According to the authors, this hypothesis is based on the "uncoupling" ability of 2,5-HD. The axon caliber is established by a three-dimensional assembly, comprising axon specific proteins that allow crossing between NF, actin and microtubules (Chiu et al., 2000). After exposure to n-hexane, due to the action of 2,5-HD, the connections between the components of this set are destroyed, because this metabolite acts in the cross-links between axon proteins, decreasing the likelihood of their occurrence (Chiu et al., 2000). More recent studies reveal that the axon atrophy involves multiple proteins of cytoskeleton, which presents the main target of the metabolite 2,5-HD (Zhang et al., 2010). They also stated that for this theory to be "accepted" and the consequences of adduct formation of cytoskeletal proteins be concretely defined, relevant targets need to be found in the amino acids residues of cytoskeletal proteins (Zhang et al., 2010).

4.3 NEUROPROTECTIVE STRATEGIES

Based on the proposed mechanisms of action of 2,5-HD and all the effects that they could cause in human health, some authors tried to propose studies focused on body detoxification, related with n-hexane exposure.

2,5-HD, as the major metabolite responsible for the neurotoxicity resulting from n-hexane exposure, can cause alterations in the body, in cells or biological processes, that could result in an overconsumption of oxygen, leading to the production of free radicals, also known as Reactive Oxygen Species (ROS). These molecules are considered highly reactive radicals that frequently attack biological molecules, by abstraction of a hydrogen atom. Among these biological molecules are lipids, whose reaction with free radicals is called lipid peroxidation (Halliwell, 1993; Kerksick and Willoughby, 2005; Mittler, 2002). This process has been associated with important pathophysiological events in a variety of diseases, drug toxicities and traumatic or ischemic injuries (Hidalgo et al., 1998). Its toxicity is based on the ability to modify protein reactive groups, producing modified proteins, called Oxidized Lipid/Amino Acid Reaction Products (OLAARPs) that could be determined. Among all the OLAARPs, only a few can be chemically determined, from which the pyrroles (Hidalgo et al., 1998), mainly compounds that result from the mechanism of action of 2,5-HD.

Following this reasoning, some authors decided to study some neuroprotective strategies to decrease or reverse the neurotoxic effects caused by 2,5-HD, which is the last metabolite of n-hexane and is responsible for the neurotoxic effects (Terenghi et al., 2011). Among several agents the N-acetylcysteine (NAC), an acetylated cysteine residue, is studied in the present work, for two reasons: its ability to maintain or increase intracellular levels of Glutathione (GSH) or to inhibit or delay cell (neuron) death (Aruoma et al., 1989; Moschou et al., 2008).

GSH is an essential compound for the attenuation of oxidative stress in cells and organs and its intracellular concentration (Sagara et al., 2010), is endogenously synthesized all throughout the body and it is basically found in all cells (Kerksick and Willoughby, 2005).

NAC has a major function through the depletion of free radicals and is a powerful antioxidant that, experimentally, has been shown to increase the GSH content in mammalian cells. Its administration to the cysteine-free medium maintains the intracellular contents of cysteine, GSH and NAC, fact that supports the possibility that NAC, after permeation into neuronal cells, is hydrolyzed to release cysteine in the cell, which is used for GSH synthesis (Sagara et al., 2010). However, in the presence of

cysteine, NAC has a deleterious effect on the cells, due to its low stability in the complete medium as compared with the cysteine-free medium (Sagara et al., 2010).

NAC also acts as a cysteine donor, being involved in the inhibition or delay of cells death, according to the concentrations administered (Moschou et al., 2008). According to some studies, some authors observed that, in small concentrations, NAC inhibits some of the ROS, which led the scientific community to ponder the increase of that concentration, in order to completely eliminate ROS (Moschou et al., 2008).

The inclusion of NAC in this study is related with the statement of some authors about the oxidation of the pyrrole ring being a critical step in n-hexane neurotoxicity. When oxidation occurs, also occurs formation of electrophilic compounds, which can react with unconjugated pyrroles, originating dimers or, otherwise, react with NH or SH groups present in proteins (Casarett and Klaassen, 2008). So, the role of NAC as an antioxidant could be important in order to reverse the formation of dimers, which originated modified proteins, responsible for the neurotoxic effects.

5 BIOMARKERS OF EXPOSURE AND OF EFFECT OF N-HEXANE

5.1 CHARACTERISTICS OF 2,5-HD BIOMARKER

In the analysis of the toxicity of n-hexane metabolites there are several biomarkers, being the most common the ones related to 2,5-HD, in blood and urine. The biomarker in urine is the most common mainly due to its way of obtaining, an easy, rapid and non-invasive method (Perbellini et al., 1993).

For the urine biomarker is important to pay attention to the form in which it is presented: free form or total form, i.e., just as the metabolite responsible for the neurotoxicity of n-hexane or as a transformation of other compounds, to reduce issues in quantitative determination. This is due to the fact that 2,5-HD has already been detected in urine samples from humans that were not exposed to n-hexane. These studies allow scientists to deduce the existence of a metabolic process, consisting in transforming the metabolite 4,5-dihydroxy-2-hexanone into 2,5-HD, by acid hydrolysis, due to a pH modification in urine (Perbellini et al., 1993).

Thus, the 2,5-HD formed in normal metabolism of n-hexane joins to 2,5-HD formed in urine by acid hydrolysis, leading to artefacts in the analysis, because the amount of 2,5-HD quantified is not proportional to the concentration of n-hexane to

which an individual has been exposed. This leads to inaccurate determinations, not fulfilling the objectives outlined in biomonitoring of n-hexane (Wang et al., 2008).

5.2 ANALYTICAL DETECTION OF PYRROLE DERIVATIVES

To have the development of the neurotoxic effects caused by exposure to n-hexane, is stated in literature that the formation of pyrrole adducts, resulting in cross-linking of proteins, through the binding between 2,5-HD and biomolecules, is an essential step for the induction of n-hexane neurotoxicity. This fact supports the importance of studying the formation of these complexes in various biological samples, besides blood and urine such as the sciatic nerve, liver, kidney, brain, etc., to try to understand the relationship between the amount of 2,5-HD and pyrrole adducts, *in vivo* (Yin et al., 2013).

These complexes not only occur between 2,5-HD and proteins, but also between any chemical that can interact with a biomolecule, making fairly wide this class of compounds. Thereby, its specific determination becomes complicated, because there are no specific tests to determine the exact type of complex formed.

The most common used method is the reaction between the pyrrole adducts and the 4-dimethyilaminobenzaldehyde (DMAB), main component of Ehrlich's reagent (EH). This reaction occurs under acid conditions (Hidalgo et al., 1998), through an electrophilic attack from the carbon atom of DMAB to the pyrrole ring. This attack forms a cation (Figure 1.5) highly conjugated that absorbs light in the visible spectrum, with a higher or lower intensity (higher or lower pink intensity) according with the pyrroles adducts concentration in the biological medium analyzed (Campbell et al., 2010; Glowaz et al., 1992; Kessler et al., 1990).

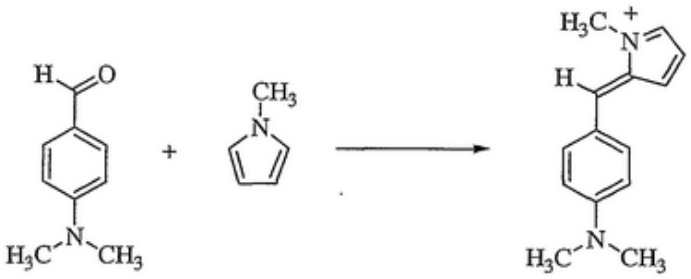

Figure 1.5: Schematic illustration of reaction between the pyrrole ring and the Ehrlich reagent. Pyrroles detection happens due to the absorption in the visible spectrum of the positive charged compound that is formed during the reaction (Brady and Robins, 2002).

OBJECTIVES

To develop this dissertation four objectives were defined:

A. Development of analytical procedures to choose the higher sensitive Ehrlich reagent and reaction conditions (namely temperature of reaction) to use in pyrrole determination;

B. Determination of all the parameters for the validation of the method used for pyrroles detection;

C. Using an *in vivo* assay, with Wistar rats repeatedly exposed to 2,5-HD, the elimination of pyrroles was studied in urine samples;

D. The protective effect of N-Acetylcysteine, as an antioxidant agent, was studied in rats co-exposed to 2,5-HD.

CHAPTER 2

EVALUATION OF THE EHRLICH SENSIBILITY IN THE DETERMINATION OF PYRROLES ADDUCTS AND METHOD VALIDATION

CHAPTER 2 – EVALUATION OF THE EHRLICH SENSIBILITY IN THE DETERMINATION OF PYRROLES ADDUCTS AND METHOD VALIDATION

1 INTRODUCTION

A test method is a process that includes manipulations susceptible of error (whether systematic or random) accumulation, responsible, in some cases, for a significant alteration in the final result. In order to reduce possible alterations, several organizations dedicated to implantation of validation methods, based on objectives tactics and criteria, conducing to the appearance of the analytical method validation (Relacre, 2000).

This validation method is one of the measurements universally recognized by laboratories, becoming a necessity for a comprehensive system of quality assurance and being an essential component of the measure that laboratories should employ to ensure that they produce accurate and reliable results (Relacre, 2000).

A method of analysis is characterized by its performance parameters, which have to be assessed if they are being used to provide the correct performance values. These performance values must be in accordance with previously defined requirements that the method of analysis should satisfy.

In the case of internal test methods, its description should be done in documents, in a detailed way, which allows anyone, with the appropriate preparation, to execute it. For this method, the minimal requirements needed for validation depend on the type of the method, comprehension of the study and knowledge of some parameters, such as linearity, working range, analytical thresholds, sensibility, precision and accuracy (Relacre, 2000).

The linearity is the ability to induce a signal or response that is directly proportional to the concentration of the given analytical parameter. It can be assessed comparing a linear and a non-linear function for each data set, through the comparison between the test-value (PG) (Equation 2.1) and the result of the Fisher-Snedecor (F) test.

$$PG = \frac{DS^2}{S^2_{y2}} \qquad \text{(Equation 2.1)}$$

where DS^2 is the difference of the variances and $S^2{}_y{}^2$ the residual standard deviation of the polynomial regression.

F test is a continuous probability distribution used to test if the variance or the standard deviation are significantly different and compare the precision of two methods. Mathematically, it corresponds to the ratio of the variances of the two data set that are being compared.

To prove that there is a linear correlation between the data set, PG needs to be smaller than F, otherwise, the function is considered non-linear and the validation is not accepted (Relacre, 2000).

Another important parameter to have in attention is the working range that corresponds to the range of concentration that can be adequately determined by the equipment, providing a useful signal that can be related to the concentration of the analyte. It is defined by applying a test of the homogeneity of variances to the data set (constituted by ten values of the first and the last standard concentration).

Mathematically, it is represented by the ratio of the two variances, being the bigger variance in the numerator (because the value needs to be higher than 1). Similar to the linearity, if this value (PG) is smaller than the F value, it means that there is no significantly difference between the variances and so the working range is well adjusted (Relacre, 2000).

The analytical thresholds are also important in validation in order to adjust as better as possible our calibration method. There are two limits: limit of detection and limit of quantification.

The limit of detection (LD) corresponds to the smallest amount of substance that can be detected in a sample, but not necessarily quantitated as an exact amount and is represented by:

$$LD = X_0 + 3{,}3.\sigma_0 \qquad \text{(Equation 2.2)}$$

where X_0 is the arithmetic mean of ten white standards and σ_0 the standard deviation associated to X_0.

The limit of quantification (LQ) corresponds to the smallest amount measured from which the analyte quantification is possible and is represented by:

$$LQ = X_0 + 10.\sigma_0 \qquad \text{(Equation 2.3)}$$

where X_0 is the arithmetic mean of ten white standards and σ_0 the standard deviation associated to X_0.

Sensibility is defined as the assessment of the method ability to distinguish small differences in the concentration of the analyte. If the calibration curve is defined by a linear regression, the sensibility will be constant and equal to the slope of that curve (Relacre, 2000).

Another important parameter is the precision that is defined as an assessment of the result dispersion between independent tests, repeated on the same sample or standards, in well-defined conditions. It could be divided into three different measurements: repeatability, reproducibility and intermediate precision. The first tests should be performed on the same sample, under very stable conditions. The second tests should also be on the same sample however the conditions should be variable. And the third one tests on the same sample, with stable conditions, varying one by one. Conditions include the laboratory, analyst, equipment, kind of reagents and duration.

These characteristics are measured based on the coefficient of variation (CV) (Equation 2.4), that needs to be smaller than 5%. In the case of the intermediate precision, the results are within three non-consecutive days (Relacre, 2000).

$$CV = \frac{S_{ri}}{\bar{x}} \times 100\% \qquad \text{(Equation 2.4)}$$

where $\bar{x}$ is the arithmetic mean of ten white standards and S_{ri} the standard deviation associated to repeatability.

The last parameter to refer to is the accuracy, which is the degree of conformity between a measurement result and the accepted reference value.

The importance of validation methods for this dissertation was related with the choice of the EH, used to detect pyrroles (due to the presence of DMAB), the main compounds responsible for the n-hexane neurotoxicity.

From the literature, it is known that there are many possible ways to prepare EH, but, based on the stability and the main goal of the use of EH, we decided to choose between two of them: one that is prepared with boron trifluoride (Kessler et al., 1990) and the other prepared with hydrochloric acid (HCl) (Campbell et al., 2010).

The studies made with both reagents, separately, included reactions at room temperature (RT) and at 45ºC, to choose the best combination between reagent and temperature that allows higher sensibility for the method, in standards and samples. This choice was made using the results from the calibration curve and then validating the chosen method.

2 MATERIAL AND METHODS

REAGENTS

The reagents that were used are: 2,5-DMP (Sigma Aldrich, USA), methanol (Merck PA, Germany), ethanol (Merck PA, Germany), 4-dimethylaminobenzaldehyde (DMAB) (Merck PA, Germany), HCl 37% (Merck PA, Germany) and boron trifluoride-methanol solution 14% in methanol (Merck PA, Germany).

PREPARATION OF EHRLICH'S REAGENTS

Two different Ehrlich reagents were prepared:

Reagent A (EH-A): 3 grams of DMAB were dissolved in a solution with 40mL of 14% boron trifluoride-methanol solution and 60 mL of ethanol (Kessler et al., 1990);

Reagent B (EH-B): 3 grams of DMAB were dissolved in a solution with 20 mL of ethanol and 80 mL of 1,25N HCl (Campbell et al., 2010; Hidalgo et al., 2004).

EQUIPMENT

For this part of the study the only equipment used was a Varian Cary 50 UV-Visible Spectrophotometer, a microplate reader, used to quantify the pyrroles concentration.

PREPARATION OF THE CALIBRATION CURVE

To prepare the solutions used to trace the calibration curve, first a 20ppm solution was prepared from the 2,5-DMP stock (ρ=0,935g/mL), then, from this 20ppm

solution, new solutions were prepared, with concentrations between 0,5ppm and 3,0ppm, in increments of 0,5ppm.

QUANTIFICATION

Absorbance was read in a Varian Cary 50 UV-Visible Spectrophotometer (microplate reader), using plates of 96 wells, each well with water, standard and EH, in the proportion of 1:3. This volume was homogenized and triplicates were measured, for both reagents, separately. In the reaction at RT, the reaction occurred in the fume-hood during 6 minutes (reaction time); at 45ºC, the reaction took place in a water-bath (6 minutes). Absorbance values were then read at 526nm.

STATISTICAL ANALYSIS

Absorbance values used for the calibration curves are expressed as means. Statistical comparisons between two different groups were made using the Mann-Whitney's test (non-parametrical test), because the number of samples was less than 10, that is the empirical minimum needed to run a parametrical test. All statistics procedures were carried out using GraphPad Software®. A p-value <0,01 was considered statistically significant.

3 RESULTS

3.1 STUDY OF THE REACTION TIME

To choose the time that gives the higher sensibility to the method we began by studying the influence of time in pyrroles adducts concentration, to a maximum of 30 minutes. Observing Table 2.1, we can see that the absorption increases until 6 minutes, decreasing after that, until 30 minutes of reaction.

Table 2.1: Influence of time in the reaction between pyrroles adducts and DMAB. The reaction took place at room temperature and using a standard solution of pyrroles.

	Time reaction (minutes)								
	2	**4**	**6**	**8**	**10**	**12**	**14**	**20**	**30**
Abs 526nm	0,5706	0,5779	0,5793	0,5768	0,5764	0,5737	0,5737	0,5692	0,5625

Abbreviations: Abs - absorbance.

3.2 STUDY OF THE INFLUENCE OF REAGENTS AND TEMPERATURE FOR PYRROLE DETECTION

After optimization of the reaction time, a study was done based on the temperature to decide if the reactions should occur at RT or at 45ºC. For both reagents, separate calibration curves were traced, one for each temperature, with the standard concentrations previous defined.

For the EH-A (Figure 2.1), we can see that at RT the method sensibility is higher than at 45ºC. However, using a statistical approach, based on the p-value (>0,01) is possible to say that there is no significant difference between the two curves.

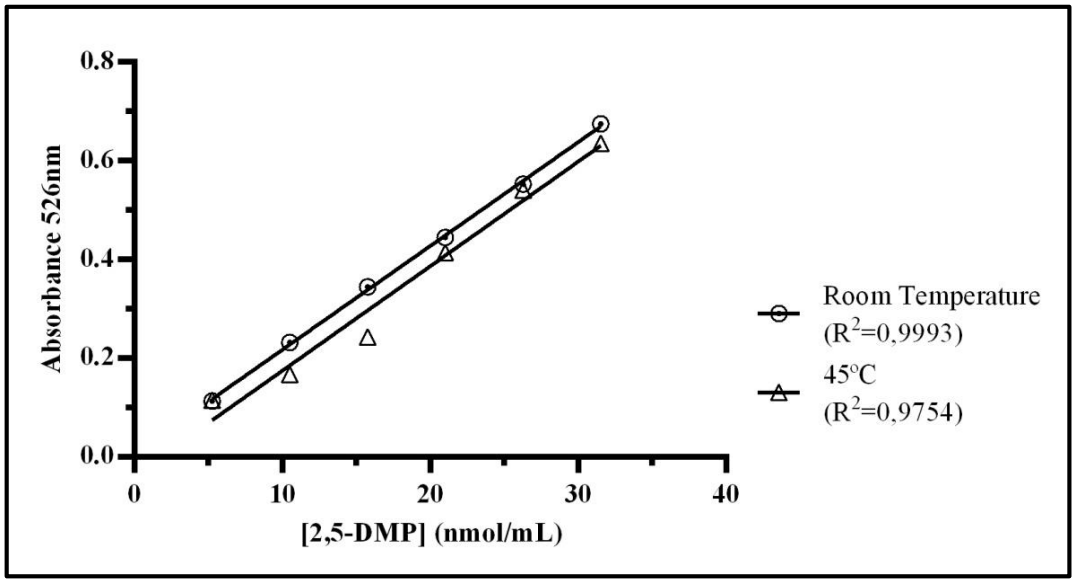

Figure 2.1: Calibration curve for the EH-A, at room temperature and at 45ºC. The curve for the room temperature is defined by y=0,02102x+0,006682; the curve for the 45ºC is defined by y=0,02117x-0,03723. From statistical comparison, p-value is 0,7879.

With the EH-B (Figure 2.2), the sensibility is also higher at RT when compared with 45ºC, but it is not statistically significant (p-value: 0,7879).

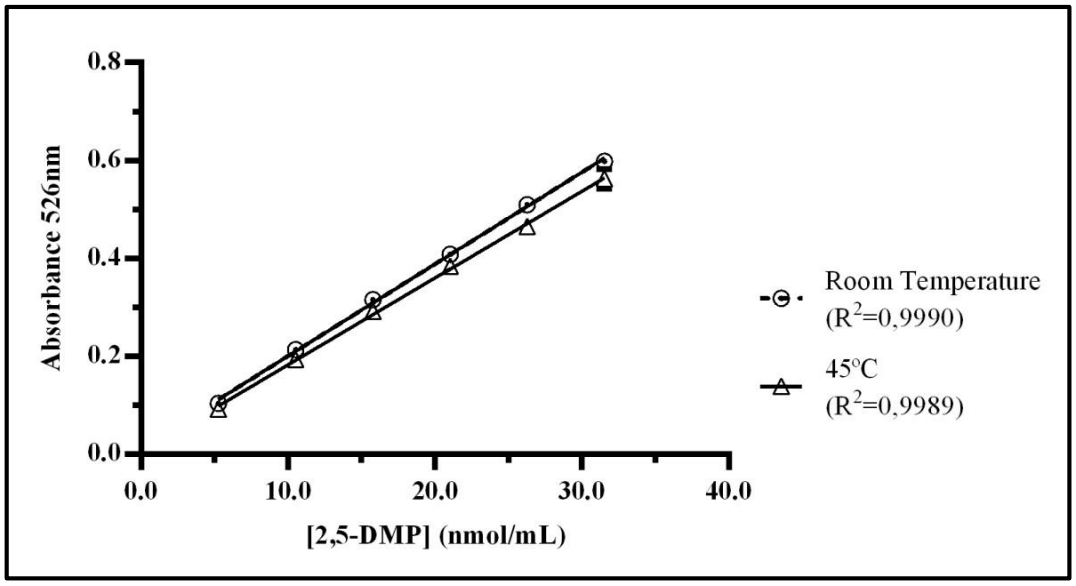

Figure 2.2: Calibration curve for the EH-B, at room temperature and at 45ºC. The curve for the room temperature is defined by: y = 0,01876x + 0,01281; the curve for the 45ºC is defined by: y = 0,01772x + 0,005254. From statistical comparison, p-value is 0,6753.

In Figure 2.3 is represented the comparison of both methods, using both reagents (EH-B and EH-A) at RT. Using a statistical approach, the sensibility was analyzed having the EH-B less sensibility when compared with the EH-A. However, statistically, these differences were not significant (p-value >0,01).

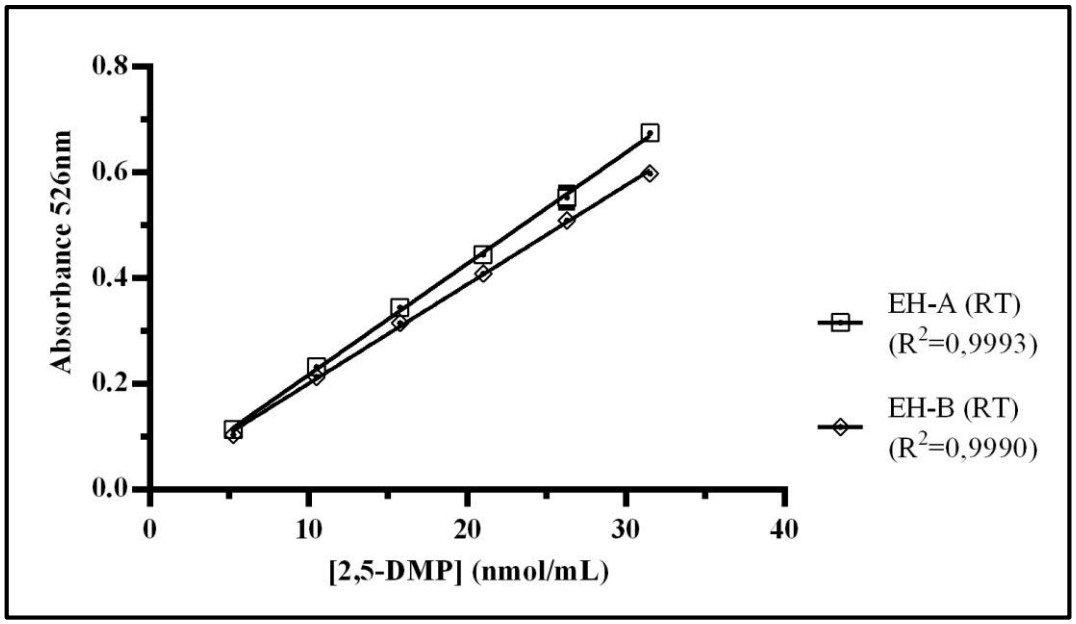

Figure 2.3: Calibration curve for the EH-A and the EH-B, at room temperature. The curve for the EH-A, at room temperature, is defined by: y = 0,02102x + 0,006682; the curve for the EH-B, at room temperature, is defined by: y = 0,01876x + 0,01281. From statistical comparison, p-value is 0,6753.

The Figure 2.4 represents the two calibration curves for both reagents, EH-B and EH-A, at 45ºC. As we can see, the data set with better correlation between the concentration and response from the equipment is the EH-B when compared with EH-A, however the sensibility is higher in EH-A. Nevertheless, statistically (p-value >0,01), there is no significant difference between them.

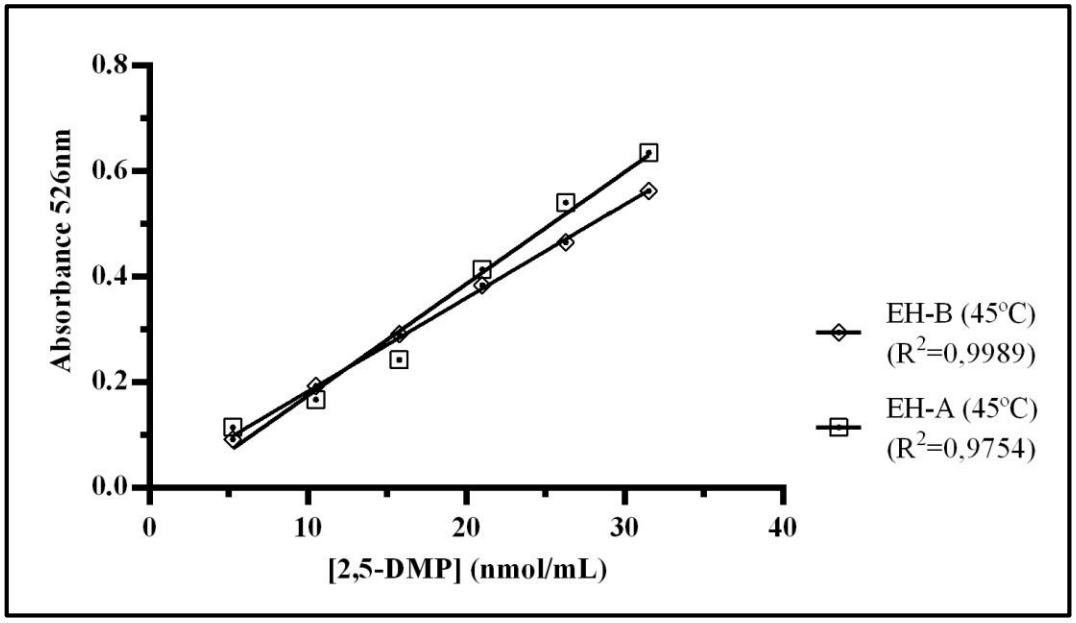

Figure 2.4: Calibration curve for the EH-A and the EH-B, at 45ºC. The curve for the EH-A, at 45ºC, is defined by: y = 0,02117x - 0,03723; the curve for the EH-A, at 45ºC, is defined by: y = 0,01772x + 0,005254. From statistical comparison, p-value is 0,8983.

3.3 METHOD VALIDATION

The validation method was applied to the EH-B and the parameter results for the linearity are presented in Figure 2.5 and in Table 2.2. Also in this table are the PG and the F values for the working range evaluation.

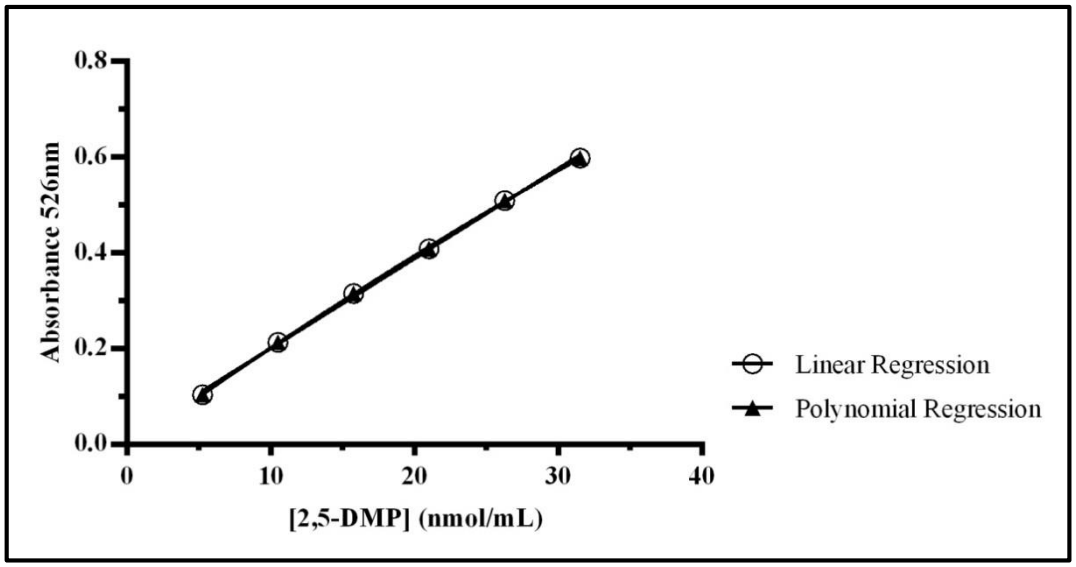

Figure 2.5: Regressions for the validation of the method. Linear Regression is defined by y=0,01876x+0,01279; Polynomial Regression is defined by y=-0,00007x2+0,02135x-0,0054.

Table 2.2: Linearity and working range. Parameters results in method validation.

Parameter	PG	F (99,5%)
Linearity	10,2741	22,7848
Working Range	2,6244	6,5411

Abbreviations: PG – test-value; F – Fisher-Snedecor test.

As we can see, for both linearity and working range, the PG value is lower than the F test, for a p-value <0,01, meaning that both parameters are well assessed, according to the parameters previous defined.

By the observation of Table 2.3, we can see that the 2,4966nmol/mL is the smallest amount of 2,5-DMP that can be detected in a sample and 3,3810nmol/mL the smallest concentration that is possible to quantify according to this method. Here is also presented the sensibility of the method.

Table 2.3: Limit of detection, quantification and sensibility. Parameters results in method validation.

Parameter	Value
Limit of Detection	2,4966 nmol/mL
Limit of Quantification	3,3810 nmol/mL
Sensibility	0,01876

Table 2.4 is a summary of the last parameters used to define the method validation. All the CV's are lower than 5%, maximum variation possible for method

validation. Exception for the repeatability in 5,255nmol/mL concentration, which difference could be associated to a less effective homogenization, before reaction.

Table 2.4: Repeatability, reproducibility and intermediate precision. Parameters results in method validation.

Parameter	CV (%)		
	5,255nmol/mL	**21,022nmol/mL**	**31,532nmol/mL**
Repeatability	6,3062%	1,3236%	2,4040
Reproducibility	Not applicable		
Intermediate Precision	3,3058	2,213	1,3897
Accuracy	Not applicable		

Abbreviations: CV - coefficient of variation.

4 DISCUSSION

Before the discussion about the chosen method to work with, it is important to explain the reason why the wavelength for the maximum absorption of the pyrrole adducts formation was 526nm and why does the reaction lasted for 6 minutes.

From the literature there is no consensus about the exact standard wavelength to measure the pyrrole adducts formation, using the EH. Depending on the target of the study, the authors trace an absorption spectrum to choose the maximum peak of absorption. According to Mattocks (Mattocks, 1967), to study unsaturated pyrrolizidine alkaloids two different wavelengths are used, based on the EH that is being used. For the EH that has boron trifluoride (EH-A) the maximum is approximately 560nm and for the EH with HCl (EH-B) is 590nm. Hidalgo (Hidalgo et al., 1998) used 590nm as the standard wavelength to determine protein damaged by oxidized lipids and 570nm for the determination of pyrrolized phospholipids (Hidalgo et al., 2004) and Kessler (Kessler et al., 1990) uses 526nm to study pyrroles formation in urine of rats.

These wavelengths are associated to different compositions of the EH and to different goals and only two of these wavelengths could be used for the purpose of this dissertation, because only those two compositions are similar to the ones used here: 570nm and 526nm. Of these two values, the one that is closer to the goal of this dissertation is 526nm, wavelength established by Kessler, because is associated with the measurement of pyrroles formation in urine samples. To improve the method, aiming higher sensibility, in the future, one thing that could be interesting to do is trace the absorption spectrum for the reaction between the pyrroles adducts and the DMAB,

using EH, just to be sure that these compounds are being measured at the maximum peak of absorption.

Another possible explication for the choice of the 526nm wavelength could be associated with the different mechanisms of pyrroles formation in the different tissues. This is due to the fact that different biological molecules behave differently, creating cross-linking with different compounds. Brady (Brady and Robins, 2001) stated that in his studies, in different studies, the pink color appears after the adding of the DMAB, suggesting that these compounds could exist in different tissues, although with different chemistries responsible for the different stabilities and therefore the different wavelength at which the absorption occurs.

About the reaction time, between the authors there are differences, depending on what is being studied. Some authors defend that the maximum absorption is reached at 5 minutes of reaction, starting with the moment of EH addition (Kessler et al., 1990; Mattocks, 1967). However, for others, the best reaction time is 30 minutes (Hidalgo et al., 1998), difference that could be associated with the high temperature. Our results support the idea of less reaction time with lower temperature, being the maximum absorption at 6 minutes of reaction.

About the results, the first parameter analyzed was the temperature, to know which temperature was the best to work with, based on the comparison of the two EH.

In Figure 2.1 is represented the EH-B at RT and at 45ºC and according to the statistical analysis, the reaction that occurs at RT has a slight more intense absorption than the reaction at 45ºC. Also, for the EH-A (Figure 2.2), the response between the concentration and the absorbance values is higher at RT. However, there is no significant difference between those data sets, which means that we could have choose one of both temperatures because the results will not present significant differences. Thus, the temperature chosen was the RT, because is the simplest and the direct way to obtain the results, when compared to the procedure done for the reaction at 45ºC.

After choosing the temperature, the following step was comparing both reagents at the same temperature, RT (Figure 2.3). Analyzing the graphical information given in Figure 2.3, EH-A has a higher sensibility and a higher correlation than the EH-B. Though, looking from a non-parametrical approach, these differences are not significant, which means that both reagents will produce very close results. According to this information and looking to the composition of both reagents, the choice was

made based on the toxicity of the substances that are in the composition of each EH. The EH-B, only includes hydrochloric acid, while the EH-A has in its composition boron trifluoride, that is a very toxic substance. That is why the EH selected for the study was the one without boron trifluoride, at RT.

The step that followed the choice of the EH, was the study of the parameters available for the validation, in order to validate the method, because if it was not validated, the samples analyses would not be trustable, due to the errors associated to the conditions that can interfere with the method, causing variability in the results.

Through Figure 2.5, is possible to see that both regressions are similar to each other and that they are well adjusted. From the point of view of the method validation, the linear regression should be the best-fit curve to those points, what corresponds to a lower PG value compared with F test. The results are in accordance with the literature, what means that this parameter could be accepted and validated. Following the same idea, we can accept the working range as well adjusted in order to validate this parameter.

The results from the thresholds limits analyses are consistent with the minimum concentration selected to the calibration curve, i.e., since the detection limit and the quantification limit are lower than the first value of the curve, there is no problem in quantifying samples using the curve defined, because what the limits are referred to are the minimum values that can be detected with this curve. So, from this approach, was possible to obtain trustable results.

Analyzing the sensibility, in this study, the only thing that is important to refer to is that this parameter assesses the method (or equipment) capability to distinguish small differences in the analyte concentrations. If the calibration curve was defined for a linear model, the sensibility will be constant throughout the working range and equal to the slope of that "curve".

Repeatability was also validated, helping to prove that all the assays were done under the same conditions, with stable parameters. Reproducibility was not applicable in this case because this was a study done for a dissertation, not to be repeated in different laboratories or with different technicians or equipment. The last parameter (intermediate precision) has a very important role in method validation because, by its analysis, it is possible to conclude that different days, different seasons (humidity, temperature), do not affect the final result.

The accuracy was not determined in this study, because reference materials are needed and those were not calculated.

CHAPTER 3

STUDY OF THE PYRROLE CONCENTRATION IN URINE OF RATS EXPOSED TO 2,5-HD AND CO-EXPOSED TO 2,5-HD+NAC

CHAPTER 3 – STUDY OF THE PYRROLE CONCENTRATION IN URINE OF RATS EXPOSED TO 2,5-HD AND CO-EXPOSED TO 2,5-HD+NAC

1 INTRODUCTION

2,5-HD is the main metabolite of n-hexane, and it is responsible for the neurotoxic effects resulting from the exposure to n-hexane. It could be assessed by measuring the concentration of pyrroles adducts in urine samples.

In this chapter, urine samples collected from rats exposed to 2,5-HD and co-exposed to 2,5-HD+NAC were assessed, to try to understand if consecutive administrations through time have any influence in the formation of pyrroles compounds.

To accomplish the main goal, urine samples were analyzed using a colorimetric assay, described in Chapter 1. Pyrroles concentrations were expressed per milligram of creatinine to correct the different urine volumes, because rats have different diuresis.

Another study done in this chapter was related with the influence of NAC dosages (in drinking water) to rats that were exposed to 2,5-HD. The aim of this study was to see if the mechanism of action would interfere with the action of 2,5-HD, based on the antioxidant characteristic that NAC shows.

To improve the sensibility of the method it was also important to study the stability of the sample during the spectrophotometric measurement. Two substances were tested: water and guanidine hydrochloride. The chosen one was used for all the spectrophotometric measurements with urine samples.

2 MATERIAL AND METHODS

REAGENTS

The reagents that were used are: 2,5-DMP (Sigma Aldrich, USA), methanol (Merck PA, Germany), ethanol (Merck PA, Germany), DMAB (Merck PA, Germany), HCl 37% (Merck PA, Germany), guanidine hydrochloride ≥99% (Sigma Aldrich, USA), 2,5-HD 99% (Fluka, Switzerland), N-acetylcysteine 99% (Sigma-Aldrich, USA), saline solution and commercial kit – CR510 for creatinine determination (Randox Laboratories, United Kingdom).

PREPARATION OF EHRLICH'S REAGENT

Reagent B: 3 grams of DMAB were dissolved in a solution with 20 mL of ethanol and 80 mL of 1,25N HCl (Campbell et al., 2010; Hidalgo et al., 2004).

EQUIPMENT

The samples were homogenized with a vortex. To separate particles in suspension, we used a Centrifuge BioFuge Pico, Heraus, with rates between 2000 and 13000 rpm. The spectrophotometer used in sample quantification was a microplate reader, Varian Cary 50 UV-Visible Spectrophotometer.

EXPERIMENTAL ANIMALS

Wistar male rats (Charles River Laboratory) were used for the *in vivo* assay. Rats were observed daily and kept under temperature and humidity controlled conditions for one week. They were then divided into four groups, according to Table 3.1. 24hours urine was collected after the 1st, 4th, 8th and 12th dosages.

Table 3.1: Experimental conditions per group. Specific conditions at which the rats were exposed, after group division.

Group	Control	2,5-Hexanedione (intraperitoneal injection)	N-acetylcysteine (drinking water)
A	Saline solution (intraperitoneal)	-	-
B	-	400 mg/ kg	-
C	-	-	200 mg/kg
D	-	400mg/ kg	200mg/ kg

All the animals were exposed to 12 dosages, on alternate days, according to the information given in Table 3.2.

Table 3.2: Amount of 2,5-HD and NAC. Amounts administrated to the rats at each dosage.

Dosages	2,5-Hexanodione (mg/kg)	N-acetylcysteine (mg/kg)
1st	400	200
4th	400	200
8th	400	200
12th	400	200

SAMPLES COLLECTION AND CONSERVATION

The animals were placed in metabolic cages to obtain the total excreted urine, in 24hours. Immediately after collection, samples were kept at -80ºC, until they were analyzed.

PREPARATION OF SAMPLES TO ANALYZE

The tube with the sample was homogenized until there was no precipitate. Then, we did tests with the samples to choose the correct dilution to apply to each sample or each group of samples, according to the calibration curve previously defined. The dilutions were then prepared and the samples were quantified. Note: All these steps should occur in the dark as much as possible, because pyrroles are highly reactive.

QUANTIFICATION

Using a 96-well plate, each well with guanidine hydrochloride 70%, urine sample and EH, in the proportion of 1:3, was homogenized. After this, the pyrroles concentration, in triplicate, were measured through the spectrophotometric method, in the microplate reader, using a wavelength of 526 nm.

STATISTICAL ANALYSIS

Absorbance values obtained for each group were used to calculate the concentration of pyrroles, based on the calibration curve presented in Chapter 2. Pyrroles compounds values are expressed as means ± SD for each group. Statistical comparisons between groups were made using the t-test with Welch's correction, a method that allows us to disregard the variances' homogeneity. All statistics procedures were carried out using GraphPad Software®. The significance level was $P < 0,01$, unless otherwise stated.

3 RESULTS

3.1 GUANIDINE STABILITY

In Table 3.3 we can see the difference in the stability of the urine from a rat exposed to 2,5-HD and a 0,5ppm standard concentration of 2,5-DMP, using water or guanidine hydrochloride for the spectrophotometric measurement.

Table 3.3: Samples stability for pyrrole detection. Stability of urine from a 2,5-HD exposed rat with guanidine hydrochloride or with water, for pyrroles detection.

Samples	Condition	Absorbance$_{526nm}$
Urine	With water	0,6163
	With guanidine hydrochloride	0,6784
5,255 nmol/ mL Standard	With water	0,1395
	With guanidine hydrochloride	0,1452

3.2 RESULTS OF EXPOSITION TO 2,5-HD

In this part of the study, there were two different groups of animals that were analyzed: group A (control group, with only saline solution via intraperitoneal injection) and group B (400mg/ kg of 2,5-HD via intraperitoneal injection). The group B was injected with 12 dosages, on alternate days, each administration with a 400mg/ kg concentration per each 48 hours (Table 3.1). Using the spectrophotometric results, the concentration of pyrroles adducts were calculated and expressed per milligram of creatinine (Table 3.4), for all five conditions (control and four dosages).

Table 3.4: Pyrrole concentration for the control and all dosages, after 2,5-HD exposure. The concentration is expressed as nanomol (nmol) of pyrrole per milligram of creatinine.

Rats Number	Control	2,5-HD dosages (nmol pyrrole/ mg creatinine)			
		1st dosage	4th dosage	8th dosage	12th dosage
1	19,2220	2224,4443	1570,2675	2036,1378	2627,1613
2	20,4343	2374,5548	1610,7315	2562,8932	2335,3355
3	18,5417	2635,2032	3539,1166	3309,0972	2421,7497
4	-	-	2756,5184	1752,6267	2765,9511
5	-	-	2746,2416	2444,3883	2578,9535
6	-	-	-	3313,3844	
Total of samples	**3**	**3**	**5**	**6**	**5**

Abbreviations: 2,5-HD - 2,5-Hexanedione.

In Table 3.4, we can see that the total number of samples in each group is different, which means that any statistical analysis using this data set would give inaccurate conclusions. So, before any conclusions were made from this data set, we needed to study, from a statistical view, if the number of samples affects the quality of the analysis. To do that, we first analyzed the original number of samples and then

analyzed all groups with the same number of samples. The chosen number was the minimum number common to all groups: 3 samples per group.

To choose which samples we were not going to consider, we made a box plot that shows the distribution of the points according to the minimum, maximum, quartiles (25% - 75%) and median (Figure 3.1). The criteria to remove samples was based on the distance from the median, i.e., the further away from the median, higher was the probability of exclusion. Table 3.5 is a summary of the samples removed per group.

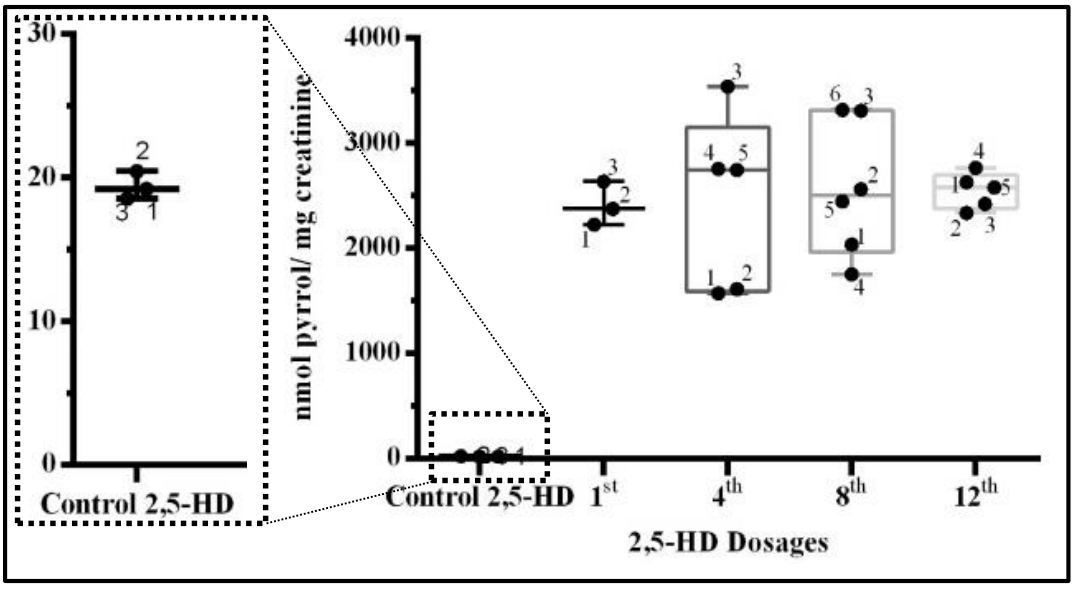

Figure 3.1: Boxplot of pyrroles concentration from the control group and 2,5-HD exposed groups. The dashed rectangle is highlighting the pyrrole concentrations values for the control group.

Table 3.5: Pyrrole concentration for the 3 samples per group of control and exposed to 2,5-HD. The concentration is expressed as nmol of pyrrole per milligram of creatinine.

Rats Number	2,5-HD dosages (nmol pyrrole/ mg creatinine)				
	Control	**1st dosage**	**4th dosage**	**8th dosage**	**12th dosage**
1	19,2220	2224,4443	-	2036,1378	2627,1613
2	20,4343	2374,5548	-	2562,8932	-
3	18,5417	2635,2032	3539,1166	-	-
4	-	-	2756,5184	-	2765,9511
5	-	-	2746,2416	2444,3883	2578,9535
6	-	-	-	-	-
Total of samples	**3**	**3**	**3**	**3**	**3**

Abbreviations: 2,5-HD - 2,5-Hexanedione.

After this, we analyzed both data sets to know if the number of samples interferes with the statistical conclusion. If it interferes, we cannot take any conclusion from the original data set. If not, we can extrapolate the conclusions from the small data set to the bigger and conclude based on the statistical analysis of the last one (the original size).

Table 3.6: Results from the statistical analysis of the difference between the control and the dosages for 2,5-HD exposure. Two populations were analyzed: one with the original samples size and the other after statistical correction (3 samples). For both, a p-value <0,01 was considered statistically different.

	Control group	
2,5-HD Dosages	**Original size population**	**3 samples size population**
1st	0,0025	0,0025
4th	0,003	0,0076
8th	0,0002	0,0047
12th	<0,0001	0,0005

Abbreviations: 2,5-HD - 2,5-Hexanedione.

Table 3.7: Results from the statistical analysis of the difference between all 2,5-HD dosages. Two populations were analyzed: one with the original samples size (left-side value) and the other after statistical correction (right-side value). For both, a p-value <0,01 was considered statistically different.

	2,5-HD Dosages							
2,5-HD Dosages	**1st**		**4th**		**8th**		**12th**	
1st			0,9366	0,1346	0,6014	0,7672	0,4026	0,1657
4th					0,7928	0,1105	0,8045	0,3060
8th							0,9332	0,1833
12th								

Abbreviations: 2,5-HD - 2,5-Hexanedione.

From Table 3.6, we can say that, looking to the analysis of the original size, all the administrations are statistically different from the control (p-value <0,01), what is supported by the analysis of the 3 samples per group, where also the p-value is lower than 0,01. Table 3.7 is related to the differences between the four dosages. There are two values for each relationship, one for the original size and the other from the analysis of 3 samples per condition. In both, all the values statistically relevant approve the hypothesis that all dosages are not significantly different (p-value >0,01) between them, what means that we can use the original pyrroles concentration values to calculate the

average of the pyrrole quantity (nmol) per milligram of creatinine for each condition (control and dosages). These calculations are presented in Table 3.8 and expressed as mean ± SD.

Table 3.8: Pyrrole compounds concentration for the groups of rats exposed to 2,5-HD, after 12 dosages.

	Control	1st dosage	4th dosage	8th dosage	12th dosage
nmol pyrrole/ mg creatinine	19,3993	2411,4007	2444,5751	2569,7546	2545,8302
SD	±0,9587	±207,8	±843,5	±643,1	±170,2

Abbreviations: SD - Standard Deviation.

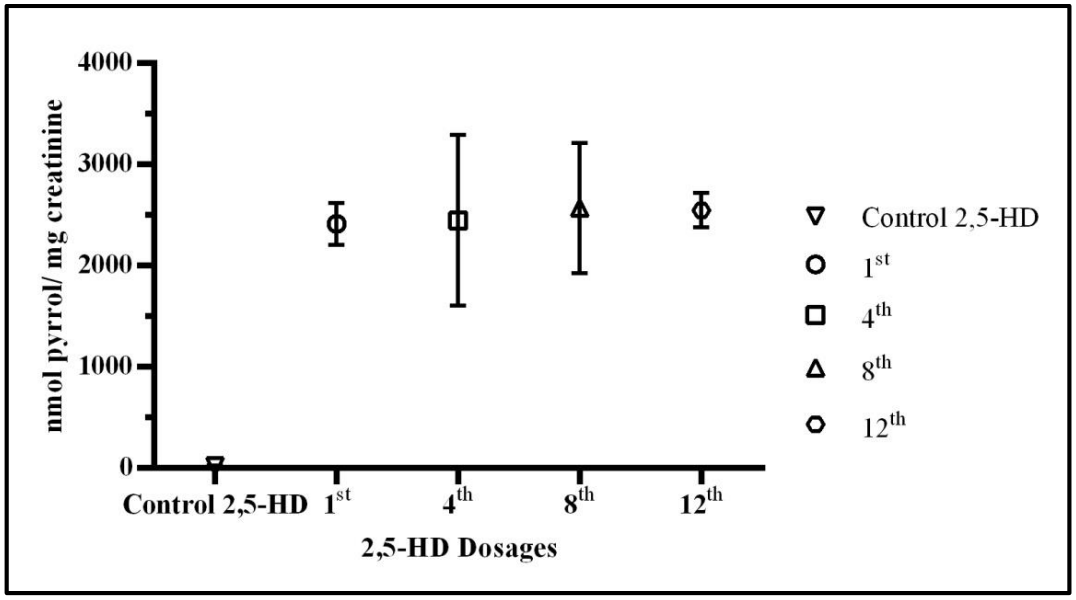

Figure 3.2: Evolution of the concentration of pyrroles during the 12 dosages of 2,5-HD. The concentration is expressed in nmol of pyrrole per milligram of creatinine and as mean ± SD.

From the simultaneous analysis of Table 3.8 and Figure 3.2, we can see that all dosages are really different from the control, being very similar between them. The second dosage has a higher concentration (when looking to mean + SD), what means that it increases from the first to the second dosage. After that, seems to occur a stabilization of the pyrrole adducts concentration, supported by the approximately constant value that the concentration has for the 8th and 12th dosages. However, if we only look to the averages, the four dosages do not have significant differences between them (Table 3.7).

3.3 RESULTS OF CO-EXPOSITION TO 2,5-HD+NAC

In this part, the other two groups were analyzed: group C (control of N-Acetylcysteine) and the group D (co-exposed to 400mg/ kg of 2,5-HD and 200mg/ kg of N-Acetylcysteine). Similar to what was done for the 2,5-HD administrations, in this section, the spectrophotometric results were used to calculate the pyrroles concentration, depending on the creatinine concentration (Table 3.9), for all conditions (control and administrations).

Table 3.9: Pyrrole concentration for the control and all dosages, after co-exposition to 2,5-HD+NAC. The concentration is expressed as nmol of pyrrole per milligram of creatinine.

Rats Number	Control	2,5-HD+NAC dosages (nmol pyrrole/ mg creatinine)			
		1st dosage	4th dosage	8th dosage	12th dosage
1	19,3641	1101,6732	2880,9620	3163,9693	2280,0428
2	15,6201	1076,4660	2683,5484	3185,4182	2084,1458
3	14,2918	2241,8089	2815,6041	3189,8427	2680,6896
4	19,6269	-	2762,5650	3220,3378	2144,0799
5	-	-	2752,1639	2440,9546	-
6	-	-	2678,4954	-	-
7	-	-	2348,2679	-	-
Group size	**4**	**3**	**7**	**5**	**4**

Abbreviations: 2,5-HD - 2,5-Hexanedione; NAC - N-acetylcysteine.

Through Table 3.9 analysis, we can see that the groups are also heterogeneous with respect to the total number of samples per condition. Because it can affect the conclusions of the statistical analysis, we also excluded the samples that were further from the median to obtain the population size with 3 samples per condition (Figure 3.3 and Table 3.10).

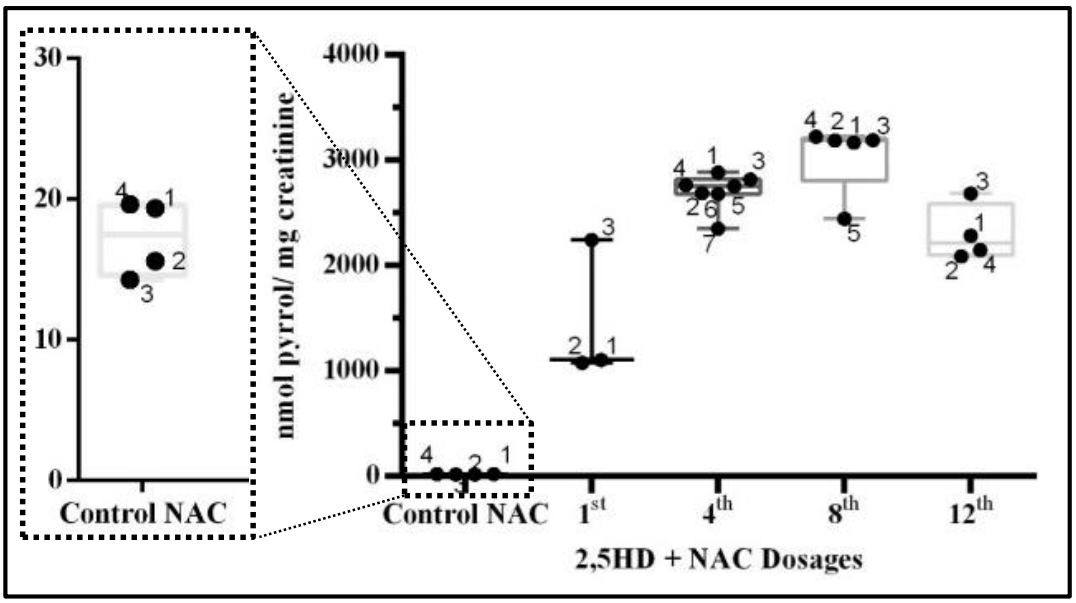

Figure 3.3: Boxplot of pyrroles concentration from the control group and 2,5-HD+NAC exposed groups. The dashed rectangle is highlighting the pyrrole concentrations values for the control group.

Table 3.10: Pyrrole concentration for the 3 samples per group of control and co-exposed to 2,5-HD+NAC. The concentration is expressed as nmol of pyrrole per milligram of creatinine.

Rats Number	2,5-HD+NAC dosages (nmol pyrrole/ mg creatinine)				
	Control	1st dosage	4th dosage	8th dosage	12th dosage
1	19,3641	1101,6732	-	-	2280,0428
2	15,6201	1076,4660	-	3185,4182	2084,1458
3	-	2241,8089	2815,6041	3189,8427	-
4	19,6269	-	2762,5650	3220,3378	2144,0799
5	-	-	2752,1639	-	-
6	-	-	-	-	-
7	-	-	-	-	-
Group size	3	3	3	3	3

Abbreviations: 2,5-HD - 2,5-Hexanedione; NAC - N-acetylcysteine.

After this sample choice, we apply a statistic test to assess if there is any difference between the two populations studied. From Table 3.11, we see that the results for the original size and the 3 sample population are identical for each data set. All dosages are significantly different from the control (p-value <0,01), except the first dosage, that, at this significance level, is not significantly different.

With respect to the comparisons between the dosages (Table 3.12), using the original size, all dosages are not significantly different among them (p-values >0,01),

except the 8th when compared with the 12th. From a 3 samples analysis, we can see that the difference between the 8th and the 12th dosages is also notable here (p-value <0,01), like between the 4th/12th, 8th/12th and 8th/12th. These differences could be associated to the SD within each group.

Table 3.11: Results from the statistical analysis of the difference between the control and all dosages for 2,5-HD+NAC co-exposure. Two populations were analyzed: one with the original samples size and the other after statistical correction. For both, a p-value <0,01 was considered statistically different.

	Control group	
2,5-HD+NAC Dosages	**Original size population**	**3 sample size population**
1st	0,0631	0,0632
4th	<0,0001	<0,0001
8th	<0,0001	<0,0001
12th	0,0004	0,0007

Abbreviations: 2,5-HD - 2,5-Hexanedione; NAC - N-acetylcysteine.

Table 3.12: Results from the statistical analysis of the difference between all 2,5-HD+NAC dosages. Two populations were analyzed: one with the original samples size (left-side value) and the other after statistical correction (right-side value). For both, a p-value <0,01 was considered statistically different.

	2,5HD+NAC Dosages							
2,5-HD+NAC Dosages	**1st**		**4th**		**8th**		**12th**	
1st			0,0814	0,0766	0,0403	0,0461	0,1545	0,2097
4th					0,0891	0,0003	0,0472	0,0049
8th							0,0078	0,0024
12th								

Abbreviations: 2,5-HD - 2,5-Hexanedione; NAC - N-acetylcysteine.

Once more, similarly to what was done with the results from 2,5-HD exposure, the average of pyrroles concentration for each condition was calculated to see how it evolves during the 12 dosages. This could be done because the differences are not notable and are most likely associated to the differences between the SD of each condition. The results are expressed as mean ± SD, in Table 3.13 and Figure 3.4.

Table 3.13: Pyrrole compounds concentration for the groups of rats co-exposed to 2,5-HD+NAC, during 12 dosages.

	Control	1st dosage	4th dosage	8th dosage	12th dosage
nmol pyrrole/ mg creatinine	17,2257	1473,3160	2703,0867	3040,1045	2297,2395
SD	±2,679	±665,7	±171,8	±335,5	±268,5

Abbreviations: SD - Standard Deviation.

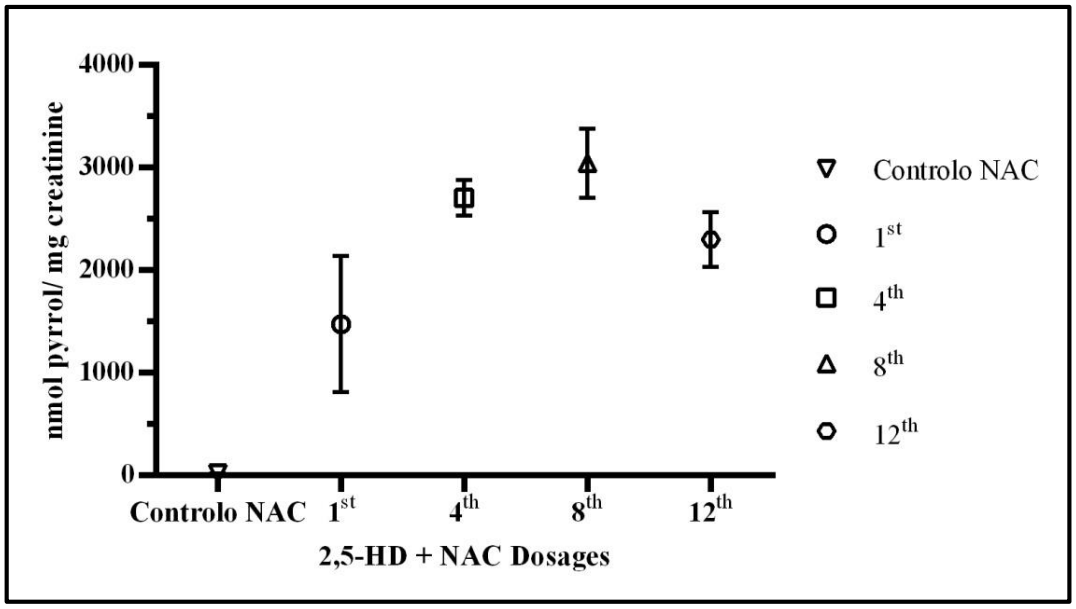

Figure 3.4: Evolution of the concentration of pyrroles during the 12 dosages of 2,5HD+NAC. The concentration is expressed in nmol of pyrrole per milligram of creatinine.

From a simultaneously analysis of Table 3.13 and Figure 3.4, we can see that all dosages are really different from the control (NAC) and slightly different between them. The 1st dosage has the lower concentration of pyrroles adducts and the 8th the highest. There seems to exist an increase in the pyrroles adducts starting with the 1st dosage until the 8th, from which the concentration starts to decrease, but still in values higher than the 1st dosage.

3.4 COMPARISON BETWEEN EXPOSITION TO 2,5-HD AND CO-EXPOSITION TO 2,5-HD + NAC

Here, co-exposed rats to 2,5-HD+NAC were studied to see if there is any experimental difference during the administration time. Table 3.14 has the summary of the pyrrole concentration for both exposure and co-exposure that is represented in Figure 3.5. Looking to this figure, we can see that the higher SD in 2,5-HD exposure

analysis is responsible for the constant behavior of pyrrole concentration during time. The samples from the co-exposed rats that were analyzed have an increasing behavior until the 12th dosage, stabilizing after that, which means that this was the group where we could see the influence of consecutive administrations.

Table 3.14: Summary of the results from exposure to 2,5-HD and co-exposure to 2,5-HD+NAC.

	2,5-HD Dosages		2,5-HD+NAC Dosages	
	N (samples)	**[pyrrole] mean ± SD**	**N (samples)**	**[pyrrole] mean ± SD**
Control	3	19,3993±0,9587	4	17,2257±2,679
1st dosage	3	2411,407±207,8	3	1473,3160±665,7
4th dosage	5	2444,5751±843,5	7	2703,0867±171,8
8th dosage	6	2569,7546±643,1	5	3040,1045±335,5
12th dosage	5	2545,8302±170,2	4	2297,2395±268,5

Abbreviations: 2,5-HD - 2,5-Hexanedione; NAC - N-acetylcysteine; SD - Standard Deviation; N – Number of samples.

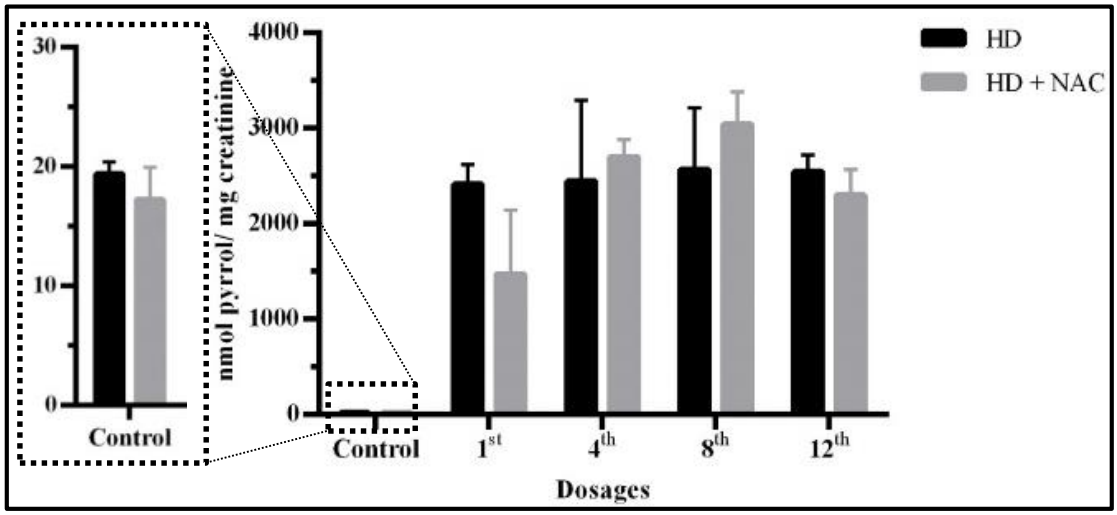

Figure 3.5: Comparison between the exposition to 2,5-HD and co-exposition to 2,5-HD+NAC. Pyrroles concentrations are expressed as means ± SD, for each group. The dashed rectangle is highlighting the pyrrole concentrations values for the control group.

4 DISCUSSION

Starting with the stability of samples, the study done with water and guanidine hydrochloride was important to understand which was the best to use in order to improve method sensibility. The choice was done by looking to the absorbance values, measured with EH-B, and we decided to use water for the calibration curve and guanidine hydrochloride for the samples. For standards we found that there was no need

in using other reagents than water, because the difference was not significant and so we do not need to any more reagents. For urine samples, we found that guanidine gives a much more stable environment compared with water. This is because this reagent has a higher capacity to unfold proteins and, since the pyrroles could mainly be associated to these biological molecules, the unfolding will free the pyrroles, allowing a more accurate quantification. This decision was also supported by some recent studies (Yin et al., 2013) that also used 70% guanidine hydrochloride for the spectrophotometric measurement of pyrroles compounds in biological samples.

Pyrroles formation is due to n-hexane exposure, which is metabolized into 2,5-HD in liver and then eliminated from the organism through urine. Then, urine samples can be analyzed to measure the concentration of pyrroles compounds to assess if there is any relation between the dosage administrated and the concentration measured.

About the exposure to 2,5-HD, we see that all administrations are significantly different from the control, which supports the idea that, once exposed, the metabolic pathway of n-hexane is activated, leading to the formation of the pyrroles adducts, responsible for the neurological alterations.

Looking only for the dosages of 2,5-HD given to the rats, we can see that there is a slight difference between them that, among other reasons, could be associated with the variation within each group caused by different metabolisms of the rats studied. For a rat with a slower metabolism, at the time of urine collection, the pyrroles concentration was probably higher in a rat that was physically incapable to handle the effects of the exposition, slowing the chemical metabolism. However, statistically, these differences were not significant, what could mean that i) the amount of 400mg/ kg is probably not enough to increase the pyrroles adducts formation at each intraperitoneal dosage or ii) the steady-state is reached. The first suggestion is supported by recent studies that based their conclusions in behavior studies and spectrophotometric measures (Yin et al., 2014). For them, a 500mg/ kg quantity of 2,5-HD do not cause any paralysis in rats and the pyrroles adducts measurement is approximately a constant line, which is only modified significantly if the amount administrated is higher than 1500mg/ kg. One thing to notice is that, in this study, 2,5-HD administrations were done by gavage and in this dissertation, that event happened through injection in intraperitoneal via. Regarding the suggestion about the steady-state, is known that at a certain time/ dosage, the absorption rate becomes similar to the elimination rate, leading to the

stabilization in the exposure curve. What could also be happening is that the amount that is being absorbed is not being totally eliminated, because the 2,5-HD could be reacting with some amino acids from other parts of the organism or some different cells that it is not taking into account at the time of the quantification.

With the co-exposition to 2,5-HD+NAC, the difference between the control group and the exposed group is statistically robust, except for the first dosage, which could be related with the above explanation for the different rats metabolism, because from a non-statistical approach we can see that there are differences between all exposed groups and control. This means that also the co-exposition activates the metabolic pathway that leads to the formation of pyrroles adducts. From the comparison between dosages, we know that they are significantly different among them, with an increasing behavior through time, compared with a constant behavior of the 2,5-HD exposure. With the last dosage, the concentration decreases, probably because of processes like auto-oxidization of pyrroles adducts, formation of stable secondary pyrroles adducts with SH groups (Yin et al., 2014).

The role of NAC in the exposure to 2,5-HD should be at the level of pyrrole ring oxidation. Due to its antioxidant characteristic, NAC could act to decrease the rate of pyrroles adduct formation or even reverse the rings already oxidized. From the presented results, we can only conclude that, effectively, for the 1st dosage, the protector effect of NAC is visible. For the following dosages, it is not possible to take conclusive observations about NAC action, because the concentration is higher than in the rats only exposed to 2,5-HD, which could mean that the amount of NAC in drinking water is not enough for the amount of 2,5-HD injected in the rats.

CHAPTER 4

FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

CHAPTER 4 – FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

1 FINAL CONSIDERATIONS

After the development of this experimental study, we may conclude:

A. Concerning the development of the analytical procedure under the study, the determination of pyrroles in urine, the influence of two different reagents with DMAB in pyrroles quantification was investigated. One of the reagents was prepared with boron trifluoride (EH-A) and the other with hydrochloric acid (EH-B). Their action in the quantification process was evaluated at two different temperatures, room temperature and 45ºC, and during different incubation times, in order to choose the best reaction time, temperature and reagent that give the higher method sensibility. After observing our, we were able to conclude that the best temperature was, definitely, the room temperature, not only for the highest absorption but also for being the easier and simplest way to do it. The reagent chosen was the one that contains hydrochloric acid, because, even that the signal was slightly lower with this one, it does not have the toxicity factor (due to the presence of boron trifluoride).

B. Thereafter, the methodology for the determination of pyrroles was validated. The linearity of the calibration curve at room temperature was evaluated, using EH-B. Based on the statistical results, we found that the linear regression was the best-fit curve to those points, what means that the parameter result could be accept and therefore validated. Also following the same type of analysis, it is possible to say that the working range is well adjusted and therefore validated. The results from the thresholds limits, when compared with the minimum concentration in the calibration curve, mean that there is no problem in sample quantification using the defined curve, because the detection and quantification limits are lower than the first value of the curve. Sensibility was a value taken from the slope of the curve that represents the ability to distinguish small differences in the analyte concentrations. The other two parameters that were also assessed, repeatability and intermediate precision, are associated with the variability that could occur if the method is done in a different laboratory, with a different equipment, different technician, different day. The results that came from the analysis of these

parameters proved that all the assays, when done under the same conditions, are not significantly different, being possible to apply this method to biological samples.

C. Before starting the analyses, the influence of the guanidine hydrochloride in pyrroles quantification related with the previous method was also investigated. Observing the results, we noticed that the absorption at 526nm was higher when guanidine was added, when compared to the results of water.

Concerning the *in vivo* assay with rats repeatedly exposed to 2,5-HD, the goal was to compare the control group (injected with saline solution) with the exposed groups, that were injected with a total of 12 dosages of 400mg/ kg every 48hours, to maximum of 12 dosages. Urine samples were selected from each group, based on the day that the urine was collected, and pyrroles concentration was measured. From the observed results, we were able to conclude that between the control and the exposed groups there are significant differences, supporting the theory of the mechanism of action of the 2,5-HD that says that once rats are exposed to n-hexane, the metabolic pathway is activated, leading to the formation of pyrrole adducts, responsible for the neurological alterations. From the comparison between the results obtained with the rats exposed to different dosages, we conclude that there is no significant difference between them, that could be associated to the variability between animals or that the steady-state was reached.

D. In the present work, we used NAC to study its possible protector effect against 2,5-HD toxicity through the determination of pyrroles in urine. The rats were exposed to2,5-HD and NAC simultaneously, through injection and in drinking water, respectively. From our results, we could see that the control (group exposed to NAC alone) is statistical different from the co-exposed groups. Between the dosages, we saw that there were differences, unlike we saw in the 2,5-HD exposure, proved by the increasing behavior through time.

The protective role of NAC is only notable in the 1st dosage, because there is a decrease in pyrrole concentration when compared to the concentration in the 1st dosage of the 2,5-HD exposure. For the others dosages, we conclude that the amount of NAC in the drinking water may not be enough for the increasing dosages of 2,5-HD. We believe that if we want to see the protector effect of NAC in all dosages, we need to

increase the amount of NAC, without forgetting the toxicity that could come from the NAC itself.

2 FUTURE PERSPECTIVES

Due to the importance that n-hexane is reaching in the society, this work could be a complement of a study about the exposure to this chemical that could be interesting for the Toxicology and Analytical Methods areas.

In this dissertation, we analyzed the effect of consecutive dosages of 2,5-HD in rats exposed via intraperitoneal to this metabolite. The results were not very conclusive, what means that to improve their quality we can either i) administrate a higher amount of 2,5-HD constant during all the administration or ii) reduce time between administrations. With this, we hope to be able to take more accurate conclusions related with effect of consecutive 2,5-HD dosages.

Also could be done a study using more biological samples. From the literature, we know that several parts of the organism are affected by the pyrroles, what means that its accumulation could occur in internal organs, such as brain, liver, sciatic nerve, and urine. The experimental work in this dissertation was done using urine samples that could be obtained from a non-invasive method. The internal organs to be analyzed will need to be obtained through an invasive method. The main goal of this study would be to assess if the measurements in non-invasive samples and in invasive methods are comparable, to know if we can evaluate an exposure based on urine samples, so it can be possible to create protocols to protect people that are exposed to n-hexane.

To finish, also the protective role of NAC to 2,5-HD is a study that could be extended. It would be very interesting if we could increase the amount given to the rats in proportion with the 2,5-HD amount, to see if it acts in the pyrroles adducts formation, decreasing or reverting these step by stopping the oxidation of the pyrrole ring. From this, we hope that from several studies, there comes a point where we can say that for a certain pyrrole adducts concentration, we need to give a certain amount of NAC.

In all these future studies that could be done, one of the most important things to notice is the needed of a higher number of rats analyzed in each group. If the number is low, like what happened in this dissertation, the results could produce unreliable results and lead to inaccurate conclusions.

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APPENDIX

APPENDIX

Table A-1: Spectrophotometric results for the control group, in 2,5-HD exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
19	0,2811	-	0,7440	19,2220
21	0,2018	-	0,4930	20,4343
22	0,2563	-	0,7000	18,5417

Abbreviations: Abs - Absorbance.

TableA-2: Spectrophotometric results for the 1st dosage group, in 2,5-HD exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
19	0,5069	1/50	0,5920	2224,4443
20	0,3184	1/100	0,6860	2374,5548
22	0,3821	1/100	0,7470	2635,2032

Abbreviations: Abs - Absorbance.

Table A-3: Spectrophotometric results for the 4th dosage group, in 2,5-HD exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
1	0,4429	1/25	0,3650	1570,2675
2	0,5954	1/25	0,4820	1610,7315
3	0,5506	1/50	0,4050	3539,1166
4	0,4927	1/50	0,4640	2756,5184
6	0,5414	1/50	0,5130	2746,2416

Abbreviations: Abs - Absorbance.

Table A-4: Spectrophotometric results for the 8th dosage group, in 2,5-HD exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
1	0,5705	1/25	0,3650	2036,1378
2	0,4763	1/50	0,4820	2562,8932
4	0,5889	1/50	0,4640	3309,0972
5	0,3587	1/50	0,5260	1752,6267
6	0,4833	1/50	0,5130	2444,3883
7	0,2699	1/125	0,5170	3313,3844

Abbreviations: Abs - Absorbance.

Table A-5: Spectrophotometric results for the 12th dosage group, in 2,5-HD exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
1	0,3913	1/25	0,1920	2627,1613
2	0,5771	1/25	0,3220	2335,3355
3	0,5453	1/25	0,2930	2421,7497
4	0,5161	1/50	0,4850	2765,9511
6	0,2795	1/125	0,6890	2578,9535

Abbreviations: Abs - Absorbance.

Table A-6: Spectrophotometric results for the control group, in 2,5-HD+NAC co-exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
8	0,2344	-	0,6100	19,3641
9	0,3518	-	1,1570	15,6201
10	0,3195	-	1,1440	14,2918
11	0,1829	-	0,4620	19,6269

Abbreviations: Abs - Absorbance.

Table A-7: Spectrophotometric results for the 1st dosage group, in 2,5-HD+NAC co-exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrol/ mg creatinine
8	0,4894	1/50	1,1530	1101,6732
9	0,5932	1/50	1,4370	1076,4660
10	0,3607	1/250	2,0680	2241,8089

Abbreviations: Abs - Absorbance.

Table A-8: Spectrophotometric results for the 4th dosage group, in 2,5-HD+NAC co-exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
12	0,3814	1/100	0,6820	2880,9620
13	0,3773	1/50	0,3620	2683,5484
14	0,4523	1/100	0,8320	2815,6041
15	0,4813	1/100	0,9040	2762,5650
16	0,2895	1/50	0,2680	2752,1639
17	0,3646	1/100	0,7000	2678,4954
18	0,4401	1/50	0,4850	2348,2679

Abbreviations: Abs - Absorbance.

Table A-9: Spectrophotometric results for the 8th dosage group, in 2,5-HD+NAC co-exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
14	0,4728	1/100	0,7750	3163,9693
15	0,4162	1/100	0,6750	3185,4182
16	0,4257	1/100	0,6900	3189,8427
17	0,3475	1/100	0,5540	3220,3378
18	0,4771	1/50	0,5070	2440,9546

Abbreviations: Abs - Absorbance.

Table A-10: Spectrophotometric results for the 12th dosage group, in 2,5-HD+NAC co-exposure. The final pyrrole concentration is expressed using creatinine concentration and using the correct dilution factor.

Rats Number	Abs_{526nm}	Dilution	Creatinine (mg/mL)	nmol pyrrole/ mg creatinine
12	0,3212	1/100	0,7210	2280,0428
14	0,3878	1/100	0,9590	2084,1458
15	0,5469	1/50	0,5310	2680,6896
16	0,4062	1/100	0,9780	2144,0799

Abbreviations: Abs - Absorbance.